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## RESULTS

## STUDIES ON PHYSIOLOGIC SPECIALIZATION OF SPHACELOTHECA SORGHI

Three years' data (1927 to 1929) have been obtained on physiologic specialization of *Sphacelotheca sorghi* at Manhattan, Kans. Eighty varieties, selections, and hybrids of the various groups of sorghums have been grown to determine their reaction to three forms of smut. Data obtained in 1929 suggest the presence of two additional physiologic forms, making a total of five (See Table 3). These physiologic forms of *S. sorghi* have been segregated on the basis of (1) the reaction of differential hosts, (2) cultural characteristics on artificial media, and (3) differences in the fragility, size, and color of the sori.

Table 1 gives the data on varietal reaction to form 1, designated as the common kernel smut; to form 2, the milo kernel smut; and to form 3, the feterita kernel smut. Blanks denote that no smut of that form or seed of the variety was available.

TABLE 1.—Percentage of smutted heads in several forms of kernel smut (*Sphacelotheca sorghi*, Manhattan, Kans., 1927, 1928, and 1929)

Group and variety, selection, or hybrid	Accession No. <sup>a</sup>	Percentage									with phvs-
		IARI									
		1927									
		1928									
		Form 1 (kafr)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafr)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafr)	Form 2 (milo)	Form 3 (feterita)	
Sorgo											
Dwarf Sumac	K. B. 2576	46 3			32 1	23 7	27 3	46 9	33 1	31 7	
Standard Sumac	K. B. 2902							40 4	13 9	64 5	
Red Amber	K. B. 2504	26 5	10 1		32 3	19 8	15 1	17 1	17 1	11 6	
Kansas Orange	K. B. 2560	47 8	23 7		20 8	17 5	34 1	26 1	25 6	38 4	
Leoti Red	F. C. I. 6610	34 7			53 2	25 5	11 3	72 2	31 3	30 1	
Freed	K. B. 2519	14 6			32 3	34 7	44 3	9 9	20 0	30 4	
Weskan	K. B. 2522				53 5	50 0	37 6	16 7	26 1	33 1	
Honey	K. B. 2574				61 2	49 7	44 3	38 6	34 9	24 2	
"Japanese Honey Drop"	K. B. 2576				7 5	7 9	8 1	29 3	25 9	27 9	
Atlas selection No. 95	K. B. 2577				96 2	68 9	34 1	20 3	33 1	34 3	
Atlas selection No. 100	K. B. 2578				84 1	53 1	50 6	11 1	35 4	20 8	
Kafr											
Reed	K. B. 2502	23 8			38 5	31 8	53 1	23 1	16 5	23 0	
Pink	K. B. 2506	36 7	49 1	50 3	65 8	37 2	33 3	13 8	50 0	27 6	
Pink selection	K. B. 2546	67 9			68 3	44 4	53 4	25 8	37 8	21 9	
Pink	F. C. I. 9091				3 0		4	15 8	35 6	39 5	
Early Pink	K. B. 2524				21 4	1 6	2 8	29 2	33 3	27 2	
Sunrise	K. B. 2523	70 8			54 8	36 4	39 6	23 3	20 2	23 8	
Blackhull	K. B. 2535	40 2	16 7		62 9	28 0	33 7	8 4	24 0	13 6	
Western Blackhull	K. B. 27102	2 2			53 2	44 2	38 1	9 5	32 7	25 7	
Dawn	K. B. 2538	20 9			57 1	45 3	40 3	23 9	26 8	20 0	
Dawn selection	H. C. 2421	68 6	33 3		51 0	39 4	32 7	12 5	24 4	16 2	
Club	H. C. 281				46 5	27 1	35 1	15 8	19 0	10 1	
Red	K. B. 2545	47 5			89 4	28 0	40 6	38 4	25 2	30 5	
Milo											
Dwarf Yellow	C. I. 332	0	10 4		0	10 7	0	0	13 2	0	
Dwarf Yellow selection	K. B. 2511	0	6 3	0	0	16 1	0	0			
Do	K. B. 2515	0	22 8		0	11 3	0	0	18 0	0	
Standard Yellow	C. I. 234		10 4		0	11 8	0	0	8 2	0	
Dwarf White	F. C. I. 8927	0	0	0	0	13 4	0	0	7 5	0	
Standard White	C. I. 352		16 8		0	9 3	0	0	7 9	0	
Cream	K. B. 2569	0	12 5	0	0	10 5	0	0	12 5	0	
Fargo Straightneck	C. I. 809	25 0	3 2	0	19 2	4 4	0	3 3	25 3	0	
"Dwarf Straightneck"	K. B. 2544				0	2 6	0	0	25 6	1 1	
"Erect"	K. B. 2545				0	19 7	0	0	32 6	0	

<sup>a</sup> The letters C. I., F. C. I., S. P. I., H. C., and K. B. indicate accession numbers, respectively, of the Division of Cereal Crops and Diseases, Division of Forage Crops and Diseases, and Division of Foreign Plant Introduction, all of the Bureau of Plant Industry, U. S. Department of Agriculture, and of the Fort Hays branch experiment station and the department of botany and plant pathology, both of the Kansas Agricultural Experiment Station.

TABLE 1—Percentage of smutted heads in sorghums inoculated with physiologic forms of kernel smut (*Sphacelotheca sorghi*) from kafir, milo, and feterita, at Manhattan, Kans., 1927, 1928, and 1929—Continued

Group and variety, selection, or hybrid	Accession No	Percentage of heads smutted after inoculation with physiologic form indicated								
		1927			1928			1929		
		Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)
Feterita										
Feterita selection.....	C I 182-1	0	0	0	0	0	0	0	0	2 1
Do.....	K B 2563	0	0	0	0	4 3	0	0	0	0 0
Feterita.....	S P I 51989	0	0	24 8	0	0	17 9	0	0	2 6
Spur.....	K B 2540	0	0	0	0	0	0	0	0	0
Red Leaf selection.....	K B 2343	0	0	0	0	4 9	0	0	0	3 9
Do.....	K B 2344	0	0	0	0	3 4	3 3	0	0	4 5
Hybrid Dwarf No 6.....	K B 2320	0	0	0	0	0	6	2 5	0	3 2
Hegari										
Hegari selection.....	K B 2518	0	2 9	0	0	8 6	0	0	1 0	0
Hegari.....	K B 2537	0	0	0	0	9 0	0	0	13 6	0
Hybrids										
Red Amber×feterita selection.....	K B 2501	0	8 2	0	0	20 5	0	0	3 4	0
Do.....	K B 2508	0	0	0	0	0	0	0	0	0
Do.....	K B 2513	0	0	0	0	3 8	0	0	0	0
Do.....	K B 2552	0	12 3	1 0	1 5	8 1	5	0	0	0
Do.....	K B 2582	0	0	0	0	0	0	0	0	0
Do.....	K B 2567	0	10 4	0	0	0	0	0	2 5	0
Do.....	K B 2570	21 5	0	15 9	25 9	0	18 1	13 8	0	21 3
Do.....	K B 2573	0	0	0	0	0	0	0	0	0
Kafir×feterita.....	F C I 8920	0	0	0	5 6	2 2	3 2	0	4 2	1 6
Do.....	H C 2423	0	0	0	0	0	18 4	0	0	0
Feterita×kafir.....	F C I 8917	0	0	0	0	0	34 4	0	0	2 4
Kafir×milo 26-3-1-1.....	K B 2561	0	34 5	10 9	0	37 0	21 8	0	26 3	13 0
Kafir×milo 35-1-2-1.....	K B 2679	42 1	0	6 3	44 3	39 3	16 4	13 2	40 8	22 5
Dwarf Yellow milo×Pink kafir.....	H C 244	52 4	0	0	51 1	52 1	51 5	0	20 3	16 3
Do.....	H C 257	0	0	0	0	26 1	12 3	0	12 8	5 6
Do.....	H C 2510	13 0	17 7	0	37 4	13 2	0	6 3	5 6	4 3
Kansas Orange×Dwarf Yellow milo selection 1.....	K B 2680	0	0	0	60 4	34 5	8 5	9 4	45 0	39 6
Kansas Orange×Dwarf Yellow selection 2.....	K B 2681	0	0	0	0	5 3	0	0	0	0
Kansas Orange×Dwarf Yellow selection 6.....	K B 2682	0	0	0	62 1	56 4	45 0	23 0	41 1	36 8
Pink kafir×Freed sorgo.....	K B 2795	56 0	0	0	50 8	26 7	53 2	23 9	43 2	45 3
Milo×feterita.....	F C I 8926	0	0	5 2	0	4	7 9	0	0	0
Blackhull×Sourless.....	K B 2503	56 0	0	0	60 7	62 9	32 7	9 4	0	0
Broomcorn										
Acme (dwarf).....	C I 243	17 8	5 0	5 3	71 5	28 8	27 5	23 0	49 2	26 5
Evergreen (standard).....	C I 583	24 7	0	0	53 6	18 2	21 6	36 5	39 2	15 1
Kaohang										
Dwarf Shantung.....	C I 293	23 9	0	0	31 8	17 5	23 9	0	26 9	21 4
Manchu Brown.....	C I 171	48 0	16 7	19 5	48 6	18 8	23 7	12 0	38 6	36 0
Miscellaneous sorghums										
White Yolo.....	K B 2525	0	40 9	0	0	60 3	0	1 2	35 3	0
Daro.....	K B 2536	5 3	5 8	19 9	3 0	31 8	36 3	0	21 4	27 6
White durra.....	C I 51	30 8	0	41 6	63 3	72 5	30 7	10 8	31 1	18 9
Shallu.....	C I 55	66 7	40 0	22 0	43 2	33 2	6 7	19 8	44 7	42 1
Shallu selection.....	K B 2579	0	0	0	75 1	15 0	7 1	17 9	50 0	22 7
Schrock.....	K B 2541	7 4	0	0	12 4	1 7	25 6	0	9 2	43 5
Modoc.....	H C 2520	0	0	0	55 4	29 1	43 5	14 6	28 3	11 4
Dwarf Freed.....	H C 2521	67 1	0	0	80 5	46 2	54 8	26 1	0	52 3
Premo.....	F C I 8929	19 4	28 1	0	17 7	33 3	0	5 8	16 7	0
Pierce kafirita selection.....	K B 2547	0	0	0	0	0	18 7	0	0	20 6
Do.....	K B 2549	52 0	0	50 6	47 2	51 8	44 2	12 8	32 9	28 4
Pierce kafirita (types 3 and 4).....	K B 27101	15 4	0	0	8 3	99 0	8 3	1 6	6 6	29 5
Sudan grass.....	K B 2981	0	0	0	0	0	0	10 0	3 5	9 0

It will be noted that the reaction of the varieties is remarkably consistent. As sorghums hybridize very readily, off-type plants occasionally occur. Also it is exceedingly difficult at times to prevent spores of the various forms of kernel smut from getting where they are not desired. This may explain the occasional occurrence of a smutted plant in a variety known to be highly resistant, and probably is accountable for the occurrence of 1.2 per cent of form 1 (kafir smut) in White Yolo, K. B. 2525. White Yolo is regarded as immune from this smut, as has been proved in all other experiments. There is, however, the possibility that the occurrence of smut in such an instance is due to the appearance of a new form of smut. In the light of present knowledge, it is possible that the occurrence of 7 smutted feterita plants in a total of 3,638 plants grown by Reed and Melchers (?) at Manhattan, Kans., Columbia, Mo., Brooklyn N. Y., and Rosslyn, Va., likewise may have been due to a similar phenomenon. The same holds true for the 3 smutted Standard White milo plants recorded by them in a total of 2,256 plants. The occurrence of physiologic forms of smut was not suspected by them at that time, however, and the logical explanation was an off-type plant or a susceptible hybrid.

In Table 2 are grouped the data on 23 varieties of sorghums selected from Table 1 and arranged to show their resistance or susceptibility to one or more of three forms of kernel smut. The reactions obtained indicated that the varieties used could be placed in seven definite groups. Kafir, broomcorn, kaohang, shallu, and Red Amber sorgo are susceptible to all three forms and are designated as group 1. Group 2 consists of Dwarf Yellow milo, hegari, and White Yolo, which are susceptible to form 2 and resistant to forms 1 and 3. Some of the Red Amber  $\times$  feterita hybrids and Spur feterita are resistant to all three forms and are placed in group 3. Group 4 consists of only one of the Red Amber  $\times$  feterita hybrids, which is susceptible to forms 1 and 3 and resistant to form 2. Most of the feteritas and some of the kaferitas are resistant to forms 1 and 2 and susceptible to form 3, and are designated as group 5. Fargo Straightneck milo and Premo belong to group 6, being susceptible to forms 1 and 2 and resistant to form 3. Group 7 consists of two selections of the milo  $\times$  kafir cross, which is resistant to form 1 and susceptible to forms 2 and 3.

In 1929 it became evident that two additional forms of kernel smut of sorghum had been found. One collection came from a feterita plant grown at the agronomy farm, Manhattan, Kans., and a second was obtained from J. H. Martin, who found it on feterita received from San Antonio, Tex., in 1927. These collections, together with the three forms previously known, were used to inoculate a limited number of sorghums, chosen as differential hosts. The results presented in Table 3 indicate that these two additional collections are distinct forms of *Sphacelotheca sorghi*. They are, therefore, designated as forms 4 and 5. Thus Dwarf Yellow milo (C. I. 332) is susceptible to form 2 and resistant to forms 1, 3, 4, and 5. White Yolo (K. B. 2525) is susceptible to forms 2 and 4 and resistant to forms 1, 3, and 5. Pierce kaferita (K. B. 2547), feterita  $\times$  kafir (F. C. I. 8917), and feterita (S. P. I. 51989) are susceptible to form 3 and resistant to forms 1, 2, 4, and 5. The kafir  $\times$  feterita hybrid (H. C. 2423) is susceptible to forms 3 and 5 and resistant to forms 1, 2, and 4.

TABLE 2.—Percentage of smutted heads in sorghums inoculated with three physiologic forms of *Sphacelotheca sorghi*, at Manhattan, Kans., 1927 to 1929, grouped according to reaction.

Group No.	Variety, selection, or hybrid	Accession No.	Percentage of heads smutted after inoculation with—								
			Form 1 (kafir)			Form 2 (milo)			Form 3 (feterita)		
			1927	1928	1929	1927	1928	1929	1927	1928	1929
1	Pink Kafir.....	K B 2506.....	36 7	65 8	18 8	49 1	37 2	50 0	50 3	33 3	27 8
	Acme broomcorn.....	C I 243.....	17 8	71 5	23 0	5 0	28 8	49 2	5 3	27 5	26 5
	Manchu Brown kaoliang.....	C I 171.....	48 0	48 6	12 0	16 7	18 8	38 6	19 5	23 7	36 0
	Shallu.....	C I 85.....	66 7	43 2	19 8	40 0	33 2	44 7	22 0	6 7	42 1
	Red Amber sorgo.....	K B 2504.....	26 5	32 3	17 1	10 1	19 8	17 1	.....	15 1	11 6
2	Dwarf Yellow milo.....	K B 2515.....	0	0	0	22 8	11 3	18 0	.....	0	0
	Do.....	C I 332.....	0	0	0	10 4	10 7	13 2	.....	0	0
	Hegari.....	K B 2518.....	0	0	0	2 9	8 6	1 0	0	0	0
	Do.....	K B 2537.....	.....	0	0	.....	9 0	13 6	.....	0	0
	White Yolo.....	K B 2525.....	0	0	1 2	40 9	60 3	35 3	0	0	0
3	Red Amber×feterita.....	K B 2509.....	0	0	0	0	0	0	0	0	0
	Do.....	K B 2562.....	0	0	0	0	0	0	0	0	0
	Do.....	K B 2573.....	0	0	0	0	0	0	0	0	0
	Spur feterita.....	K B 2540.....	0	0	0	0	0	0	.....	0	0
4	Red Amber×feterita.....	K B 2570.....	21 5	25 9	13 8	0	0	15 9	18 1	21 3	.....
5	Red-leaved feterita.....	K B 2543.....	0	0	0	0	0	0	.....	4 9	3 9
	Feterita.....	S P I 51989.....	0	0	0	0	0	24 8	17 9	2 6	.....
	Pierce kaferta.....	K B 2547.....	0	0	0	0	0	0	18 7	20 6	.....
	Feterita×kafir.....	F C I 8917.....	.....	0	0	0	0	0	.....	34 4	2 4
6	Fargo Straightneck milo.....	C I 809.....	25 0	19 2	3 3	3 2	4 4	25 3	0	0	0
	Premo.....	F C I 8929.....	19 4	17 7	5 8	28 1	33 3	16 7	0	0	0
7	Dwarf Yellow milo×Pink kafir.....	H C 257.....	0	0	0	0	26 1	12 8	.....	12 3	5 6
	Kafir×milo 26-3-1-1.....	K B 2561.....	0	0	0	34 5	37 0	26 3	10 9	21 8	13 0

TABLE 3.—Percentage of smutted heads in sorghums inoculated with five physiologic forms of *Sphacelotheca sorghi*, at Manhattan, Kans., 1929

Variety, selection, or hybrid	Accession No.	Percentage of heads smutted after inoculation with—				
		Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 4 (feterita)	Form 5 (feterita)
Dwarf Yellow milo.....	K B 2515.....	0	18 0	0	0	0
Do.....	C I 332.....	0	13 2	0	0	0
White Yolo.....	K B 2525.....	1 2	35 3	0	12 6	0
Pierce kaferta.....	K B 2547.....	0	0	20 6	0	0
Feterita×kafir.....	F C I 8917.....	0	0	2 4	0	0
Feterita.....	S P I 51989.....	0	0	2 6	0	0
Kafir×feterita.....	H C 2423.....	0	0	18 4	0	41 6

\* Percentage of smut obtained in 1928. On account of shortage of seed there are no 1929 data on the reaction of these two forms.

For aid in identifying the five physiologic forms of kernel smut (*Sphacelotheca sorghi*) the various groups of sorghums used to differentiate them are arranged in the following dichotomous key

## KEY FOR THE IDENTIFICATION OF PHYSIOLOGIC FORMS OF SPHACELOTHECA SORGHI

- A Kafir × feterita (H. C. 2423), resistant  
 B. Dwarf Yellow milo (C. I. 332), highly resistant  
   C White Yolo (K. B. 2525), resistant..... Form 1  
   CC Yolo (K. B. 2525), susceptible..... Form 4.  
 BB. Dwarf Yellow milo (C. I. 332), moderately susceptible..... Form 2  
 AA. Kafir × feterita (H. C. 2423), susceptible.  
   B. Pierce kaferta (K. B. 2547), feterita × kafir (F. C. I. 8917),  
     and feterita (S. P. I. 51989), highly resistant..... Form 5.  
   BB. Pierce kaferta (K. B. 2547), feterita × kafir (F. C. I. 8917),  
     and feterita (S. P. I. 51989), susceptible..... Form 3.

## MORPHOLOGIC CHARACTERS OF SPHACELOTHECA SORGHI

Ficke and Johnston (1) have shown that forms 1, 2, and 3 of *Sphacelotheca sorghi* may be separated from one another when grown on various artificial culture media, by the color, surface contour consistency, margin, and rate of growth of the colonies. They also noted that sectoring was rather common in form 1, but rarely occurred in form 2 and was not observed at all in form 3. The cultural characteristics seemed, according to these authors, to be fairly constant. Their studies did not consider the possible morphologic differences of the fungus other than those exhibited in culture. Observations by these authors in the field did not indicate any consistent difference in shape, size, or color of the sori.

TABLE 4.—Data on approximate length, color, and degree of rupturing of sori of five physiologic forms of *Sphacelotheca sorghi* in two series of smutted panicles of varieties and strains of sorghum, at Manhattan, Kans., 1929

Physiologic form No.	Series	No. of smutted panicles	Average protrusion of sor	Varieties with membranes of indicated color		Average degree of rupturing of membrane <sup>a</sup>
				Brown	White	
			Mm.	Number	Number	
1.....	{	1	3.7	38	0	0.0
		2	3.8	35	0	0.7
2.....	{	1	4.0	20	18	2.7
		2	3.7	21	16	2.9
3.....	{	1	5.4	2	35	7.7
		2	5.3	1	35	5.5
4.....	{	1	3.4	39	0	9.9
		2	3.8	36	0	4.4
5 <sup>b</sup> .....	{	1	4.1	12	4	4.4

<sup>a</sup> In recording the degree of rupturing of the membrane of the sori, the material was divided into five classes represented by the following units of measurement: 0=No rupturing, 1=very few sori ruptured and ruptured only to a slight extent, 2=few sori ruptured to a moderate extent, 3=many sori ruptured to a moderate extent, and 4=most sori greatly ruptured.

<sup>b</sup> Only 16 varieties were included, and 15 of the 16 panicles were only partially smutted.

In the present studies the sori of all five physiologic forms studied were carefully examined, both the chlamydospores and the membranes, as well as the general form and rupturing. Large numbers of chlamydospores of each form were measured. All measurements fell within the range given for *Sphacelotheca sorghi*, namely, 5 $\mu$  to 9 $\mu$ . The spores of all forms were found to be dark brown in color when observed in mass and olive brown when observed singly. Spores of all forms also were observed to be spherical or subspherical and rather thick-walled and smooth. The characteristics of the membrane of the sori were studied in some detail by choosing a typical smutted head of each of the five physiologic forms occurring on 43 different varieties or selections of sorghum and placing them on a table arranged by variety and smut form. The smutted panicles were from the same varieties as were used in the inoculation studies previously discussed. For these studies of the sori an effort was made to choose varieties that were susceptible to all five physiologic forms of the smut. Thus it was possible to compare the five forms on a number of the same varieties of sorghum. In the case of form 5, however, only 16 varieties developed smut. Observations were made on the approximate length, color, and rupturing of the sori of all five forms of smut. Both macroscopically and microscopically the sori were found to be typically

those of *S. sorghi* and did not resemble those of *S. cruenta*. After the first series of panicles had been examined and the results recorded, a second series of typical smutted heads was chosen and similarly compared. The results, as given in Table 4, are based on the two sets of observations.

The relative length of the sori of each smutted panicle was recorded as long, medium, or short. The protrusions of the sori beyond the glumes for these three classes were Long, 5 to 7 mm, medium, 3 to 5 mm, short, 1 to 3 mm. Although no actual measurements were made, the range is so great, especially for the two extreme classes, that it is not likely that any error in classification occurred. In

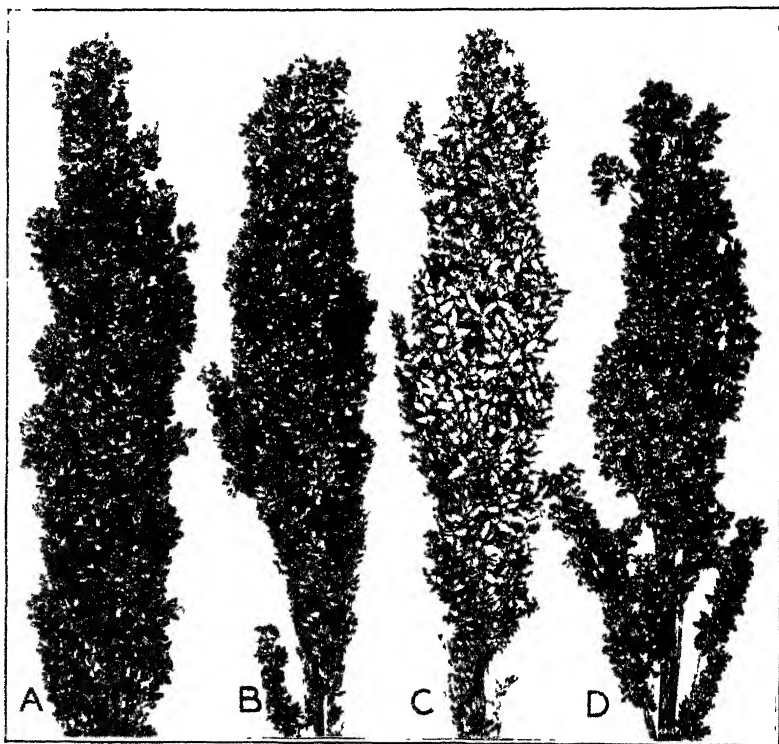


FIGURE 1—Physiologic forms of *Sphacelotheca sorghi* on Pink kafir. A, Physiologic form 1, membranes of sori brown and mostly unruptured, sori short. B, Physiologic form 2, membranes of sori may be white or light brown, badly ruptured, spores escaping, sori moderately short. C, Physiologic form 3, membranes of sori white and mostly unruptured, sori very long. D, Physiologic form 4, membranes of sori brown and unruptured, sori moderately long.

summarizing the data for Table 4, the number of individuals in each class was multiplied by the median of the class, the results added and divided by the total number of measurements. This gave what appeared to be a reasonable figure for the average length of the sori for each physiologic form. The color of the membranes of the sori was recorded as brown or white, the total number of smutted panicles showing each color for each physiologic form being given in the table.

It will be observed from the data in Table 4 that forms 1 and 4 are characterized by membranes that are brown in color and only

slightly ruptured. The sori are intermediate in length. The membranes of form 2 are either brown or white, depending on the variety, and usually they are considerably ruptured. Form 3 is characterized by long sori with white membranes that are seldom ruptured to an appreciable extent. In form 5 the sori are rather large and may have either brown or white membranes, with little rupturing. It is also characteristic of form 5 to produce only partial smutting of the panicles. Specimens of Pink kafir attacked by four different physiologic forms are shown in Figure 1, and Manchu Brown kaoliang attacked by form 5 is shown in Figure 2.

From the data at hand it is doubtful whether the five physiologic forms of *Sphacelotheca sorghi* can be separated solely on the basis of observable differences in the sori. This has not been attempted over a series of years or under different environmental conditions. However, the data in Table 4 indicate several striking differences that may be helpful in making tentative identifications.

#### DISTRIBUTION AND IMPORTANCE OF PHYSIOLOGIC FORMS OF *SPHACELOTHECA SORGHI*

Observations and records over a period of years show that the kafir kernel smut (form 1) is most common and widespread in the United States. Form 2 is less common, but since it attacks many varieties that

are susceptible to form 1 it may possibly become more common and become a problem in areas devoted to the milo crop. Such is the case in parts of New Mexico and Texas, and undoubtedly will be

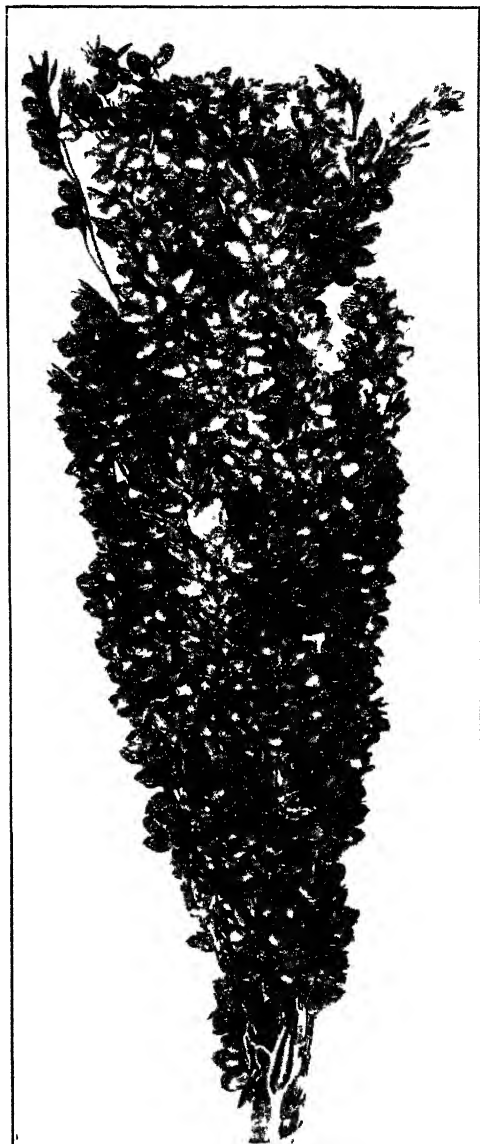


FIGURE 2.—Physiologic form 5 of *Sphacelotheca sorghi* on Manchu Brown kaoliang. A few florets in the tip of the head are smutted, while the remainder of the panicle is unsmutted. This reaction to physiologic form 5 is typical.

true in Kansas and other States where milo is an important crop. Less is known at the present time about the performance and distribution of forms 3, 4, and 5, although the possibility of their increasing is an important consideration from the standpoint of breeding for resistance to the kernel smut of sorghum.

The occurrence of physiologic forms of kernel smut of sorghum that attack hitherto resistant or immune varieties and strains has greatly complicated the problem of breeding for resistance to kernel smut. Smut-resistant hybrids produced several years ago at the Kansas Agricultural Experiment Station are now susceptible to one or more of the forms of sorghum smut, thereby reducing the number which formerly were regarded as immune.

Seed-treatment experiments (2, 4, 5) conducted over several years have shown that sorghum kernel smut (forms 1 to 5) is effectively controlled by the copper-carbonate dust method. This method is almost exclusively used in Kansas because of its efficiency, ease of application, and cheapness.

#### SUMMARY

Three years' data on physiologic specialization of *Sphacelotheca sorghi* have been obtained. Eighty varieties, selections, and hybrids comprising the various groups of sorghums have been used in testing five physiologic forms.

These forms have been designated as forms 1, 2, 3, 4, and 5. They may be separated by the reactions of varieties of sorghums.

There are no outstanding morphologic differences between the chlamydospores of the five physiologic forms of *Sphacelotheca sorghi* studied.

One year's data from comparative studies of the morphologic characteristics of the sori of the five physiologic forms of *S. sorghi* on a number of sorghum varieties have shown some rather definite differences in length, color, and rupturing.

Varieties such as durra, milo, selections of feterita, darso, Dwarf hegari, White Yolo, and certain hybrids, which a few years ago were known to be highly resistant to or immune from *Sphacelotheca sorghi* infection, are now known to be somewhat susceptible to one or more physiologic forms. Of the varieties, selections, and hybrids so far grown, 1 selection of Spur feterita and 3 Red Amber  $\times$  feterita crosses remain immune from all 5 forms of smut. The sorghum host range used in these studies, however, has not been exhausted.

The occurrence of physiologic forms of smut that attack hitherto resistant or immune sorghums has greatly complicated the problem of breeding for resistance to kernel smut.

There is evidence that form 1 is most common and most widely distributed in sorghum-growing areas of the United States. Form 2 is less common. Less is known about the occurrence and distribution of forms 3, 4, and 5.

Seed-treatment experiments conducted at Manhattan, Kans., have shown that forms 1 to 5 may be controlled by the copper-carbonate dust seed treatment.

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# INHERITANCE OF HEIGHT IN BROOMCORN<sup>1</sup>

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## INTRODUCTION

Broomcorn (*Holcus sorghum* L. *Sorghum vulgare* Pers.) is a crop of which both the varietal and commercial classes are based upon differences in plant height. The varieties of broomcorn that are, or have been, produced commercially in this country are divided on the basis of height into three groups—standard (tall), western dwarf, and whisk dwarf.<sup>2</sup> Records show that broomcorn of the standard type was imported into the United States at an early date, while the western dwarf and whisk dwarf types appeared later, with no evidence of foreign introduction. The broomcorn dwarfs thus far observed have had shorter internodes but practically the same number of nodes as the standard type. This fact has been so apparent (figs. 1 and 2) that the evidence of detailed counts of the number of nodes is not considered necessary.

In the course of varietal improvement crosses were made between varieties of the several types of broomcorn. The unexpected occurrence of plants of standard height in a cross between two dwarf types indicated the need for a study of height inheritance in broomcorn. This paper gives the results of such an investigation.

## CROSSES STUDIED

Crosses were made at the United States Dry-Land Field Station, Woodward, Okla., in 1919, between a strain of standard broomcorn (Evergreen, C I<sup>3</sup> No. 556) and a western dwarf variety (Acme, C I No. 243), and also between a whisk dwarf variety (Japanese Dwarf, C I No. 442) and the Evergreen variety. The average heights of the three parental varieties in an 8-year period were: Evergreen, 97 inches; Acme, 57 inches; Japanese Dwarf, 42 inches. The crossed seeds were planted and produced F<sub>1</sub> plants in 1920. In 1921 two 8-rod rows of the F<sub>2</sub> generation of the cross between Evergreen and Acme were grown and height data were obtained. Measurements of the parental varieties in adjoining rows also were obtained.

A number of broomcorn crosses were again made in 1923 to study the inheritance of the broomcorn height factors. These included Acme × Evergreen, Japanese Dwarf × Acme, and Japanese Dwarf × Evergreen. It was expected that height data could be obtained from the F<sub>2</sub> generations of these crosses in 1925. The season, however, was unfavorable for normal height development of most broomcorns, especially the standard varieties, and consequently height measurements were not taken.

In 1927 to obtain additional data a cross was again made between the Acme and Japanese Dwarf varieties.

<sup>1</sup> Received for publication June 30, 1931; issued February, 1932.

<sup>2</sup> SIEGLINGER, J. B. BROOMCORN EXPERIMENTS AT THE UNITED STATES DRY-LAND FIELD STATION, WOODWARD, OKLA. U. S. Dept. Agr. Tech. Bul. 51, 32 p., illus. 1928.

<sup>3</sup> C I refers to accession number of Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

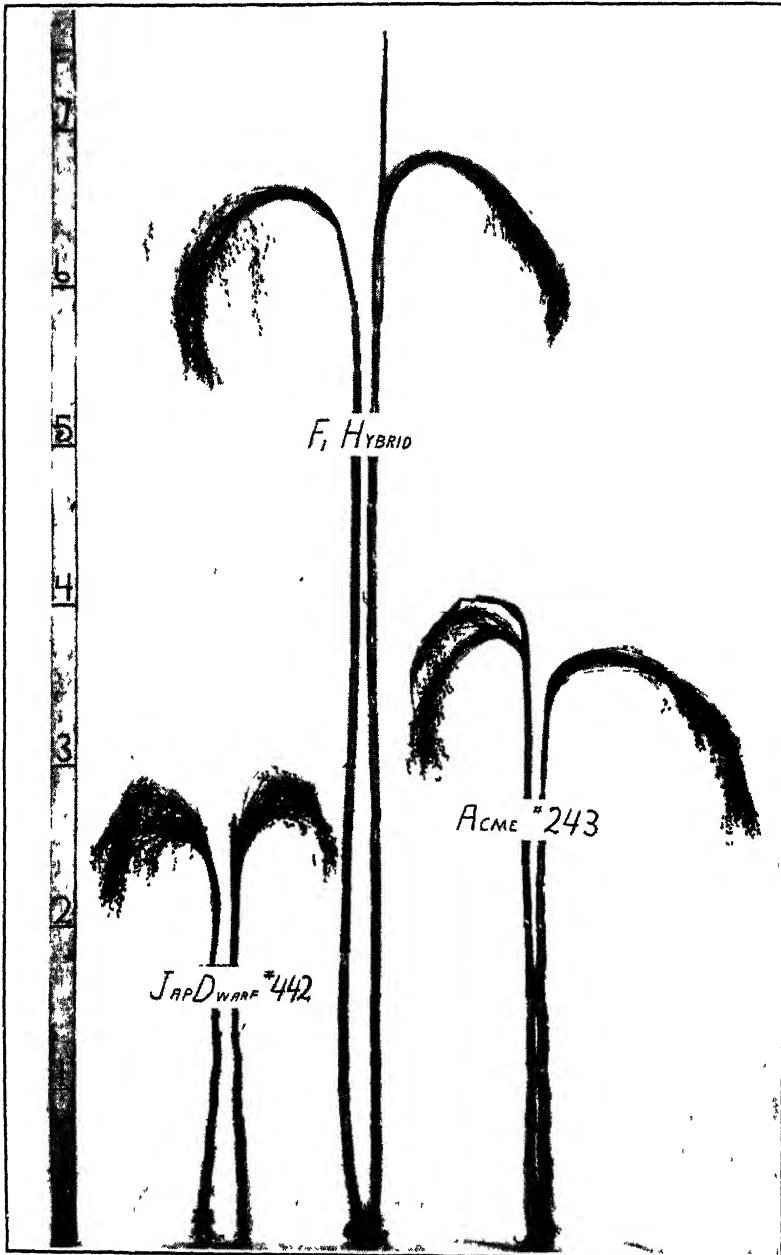


FIGURE 1—Plants of the  $F_1$  cross Acme  $\times$  Japanese Dwarf broomcorn, and the parents



FIGURE 2—Stalks representative of the four phenotypes of the  $F_2$  cross, Acme  $\times$  Japanese Dwarf broomcorn (Parent plants at left)

## RESULTS

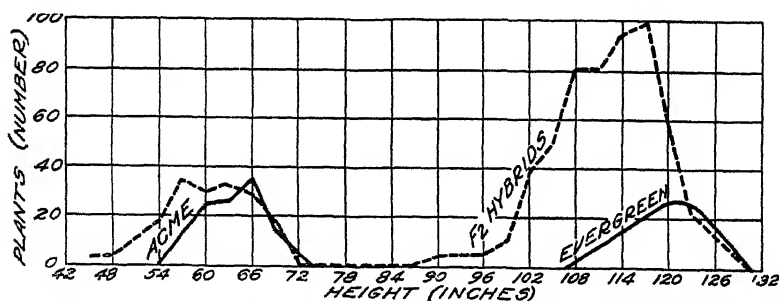
STANDARD  $\times$  DWARF CROSSES

The average heights of the  $F_1$  plants in comparison with the dwarf and standard parents are shown in Table 1. It is realized that these numbers are too small to be statistically reliable, but it is apparent that the  $F_1$  plants were about as tall as the Evergreen (standard) parent. This shows a dominance of the standard height.

TABLE 1 — Height of parents and  $F_1$  broomcorn crosses in 1924

Variety	Group	Number of plants	Height
			Inches
Evergreen.....	Standard.....	3	103 $\pm$ 0.1
Acme.....	Western dwarf.....	4	62 $\pm$ 6
Japanese Dwarf.....	Whisk dwarf.....	6	41.8 $\pm$ 8
Japanese Dwarf $\times$ Evergreen.....		1	99
Acme $\times$ Evergreen.....		4	101.5 $\pm$ 1.6

The results from the  $F_2$  progenies in 1921 (fig. 3) showed a simple 3:1 segregation of standard and western dwarf types in the cross of Evergreen  $\times$  Acme and its reciprocal. Of the 767 plants, 582 were of the standard type, averaging  $112.0 \pm 0.20$  inches in height, and 185 were of the western dwarf type, having an average height of  $60.5 \pm 0.3$  inches.

FIGURE 3 — Height of  $F_2$  plants of a broomcorn cross, Evergreen  $\times$  Acme, and of the parent varieties, 1921

The adjoining rows of the parental Evergreen variety averaged  $119.6 \pm 0.3$  inches, and Acme averaged  $63.8 \pm 0.26$  inches in height. There was no difficulty in classifying the two  $F_2$  types, as is evident from Figure 3.

The following year, 1922, 12 rows of the Evergreen  $\times$  Acme cross were grown from selfed  $F_2$  heads. Six of these rows, from plants classed as dwarf in the  $F_2$  generation, produced only dwarf plants in the  $F_3$  generation. Of the six rows from plants classed as standard in the  $F_2$  generation, five segregated for standard and dwarf plants and one was pure for standard height.

The Acme  $\times$  Evergreen cross in 1925 again produced standard and western dwarf types in the  $F_2$  generation in the ratio of 3:1, thus confirming the 1921 results on this cross.

The above results show that the standard (tall) type of broomcorn differs from the western dwarf type by a single height factor.

WHISK DWARF  $\times$  WESTERN DWARF CROSSES

The average height, in 1924, of 12  $F_1$  plants of a cross between Japanese Dwarf (whisk dwarf) and Acme (western dwarf) broomcorn was  $101.4 \pm 0.8$  inches. In this experiment six Japanese Dwarf plants averaged  $41.8 \pm 0.8$  inches and four Acme plants averaged  $62 \pm 0.6$  inches in height. Plants of the  $F_1$  hybrid and the two parents are shown in Figure 1. Evergreen (standard) plants under the same conditions averaged  $103 \pm 0.4$  inches in height, or almost the same as the  $F_1$  plants from the cross between the two dwarf varieties. This indicates the presence of complementary height factors in the two dwarf types.

The  $F_1$  plants of the above cross grown in 1928 averaged 86 inches in height, as compared with 53 inches for Acme and 40 inches for the Japanese Dwarf parent.

The Japanese Dwarf  $\times$  Acme cross produced plants in the  $F_2$  generation, in 1925, corresponding in height to the  $F_1$  (standard) plant and to each of the dwarf plants. In addition there appeared a fourth class of extremely short or "double dwarf" plants (Fig. 2). The plants of the dwarf groups could not all be accurately classified for height, however, because of the unfavorable growing conditions in 1925.

The double dwarf type of plant is not represented by any commercial variety of broomcorn. Extra dwarf or double dwarf plants have been observed, however, in other sorghum groups, particularly in milo, of which the Double Dwarf variety is extensively grown. The double dwarf type of broomcorn was first observed in 1921 in the  $F_2$  progeny of a natural hybrid plant of standard height found in a field of Japanese Dwarf broomcorn. The 275 plants of this natural hybrid population segregated into 167 standard plants, 47 western dwarf, 48 whisk dwarf, and 13 double dwarf plants, closely approaching a di-hybrid ratio. A true breeding strain of double dwarf broomcorn from this cross has been maintained since 1921.

Remnants of seed from  $F_1$  plants of the Japanese Dwarf  $\times$  Acme cross were sown in 1926, but they germinated poorly. Five 8-rod rows contained a total of only 196 plants. These were measured and classified, and observed groupings are compared in Table 2 with the calculated numbers based on a 9:3:3:1 ratio. This distribution gives a  $\chi^2$  value of 1.97, with  $P=0.579$ , which indicates that a deviation as great as or greater than that observed may be expected to occur about six times in 10 as a result of random variation.

TABLE 2 — *Broomcorn plants from  $F_1$  seed measured and classified, with calculated numbers based on a 9:3:3:1 ratio*

Phenotype	Observed	Calculated	Mean height
			<i>Inches</i>
Standard.....	115	110.25	95.8
Western dwarf.....	40	36.75	57.2
Whisk dwarf.....	32	36.75	44.4
Double dwarf.....	9	12.25	24.9

In 1929 five 8-rod rows of the  $F_2$  generation produced 525 plants. These and a number of plants of the parent varieties were measured for height. Although the development of the  $F_2$  plants was fairly

satisfactory, there was not a clear-cut difference between the three dwarf classes in 1929 but a partial blending from one height class to the next. (Fig 4) There was no overlapping between the height of western dwarf and that of standard, though the difference in height was less pronounced than it would have been had the plants been grown under better environmental conditions. The measurements of the parent and  $F_2$  populations and the segregation of the latter are shown in Table 3 and Figure 4.

TABLE 3—Height of parents and  $F_2$  plants of a cross between whisk dwarf and western dwarf broomcorn in 1929

Height class	Total plants	Range in height	Mean height	Standard deviation	Coefficient of variability
Parents	<i>Number</i>	<i>Inches</i>	<i>Inches</i>		
Whisk dwarf.....	45	32-45	39 $0 \pm 0$ 3	3 22	8 25
Western dwarf.....	53	49-64	57 $6 \pm$ 3	3 20	5 56
$F_2$ hybrids					
Double dwarf.....	34	13-31	24 $6 \pm$ 5	4 58	18 62
Whisk dwarf.....	94	32-47	40 $5 \pm$ 28	4 08	10 07
Western dwarf.....	104	48-68	55 $6 \pm$ 29	4 44	7 99
Standard.....	293	73-112	91 $4 \pm$ 29	7 39	8 08

About 200 of the  $F_2$  plants were selfed, and in 1930 progenies from 112 typical plants, representing all height classes, were grown in head

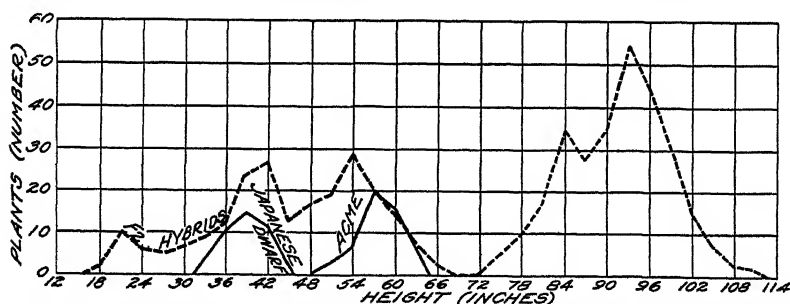


FIGURE 4—Height of  $F_2$  plants of a broomcorn cross (Acme  $\times$  Japanese Dwarf) and of the parent varieties, 1929

rows to observe their genotypic behavior in the  $F_3$  generation. The study of these  $F_3$  progenies showed that the classification of the plants for standard height in the  $F_2$  generation was correct. All of the 44 standard  $F_2$  plants produced some standard plants in the  $F_3$  generation. There was a discrepancy in classifying the double dwarfs because several of the very short plants produced heterozygous whisk dwarf progenies in the  $F_3$  generation. Also, a few  $F_2$  plants that were classed as western dwarf proved to be whisk dwarf. Based on the proportions observed and the behavior of the 112  $F_3$  progenies produced in 1930, the corrected numbers in the entire  $F_2$  population of 525 plants were as shown in Table 4.

This distribution gives a  $\chi^2$  value of 6.11 and a  $P$  value of 0.108. A fit as poor as the foregoing may be expected once in nine or ten times as a result of random variation.

TABLE 4—Observed and calculated numbers from entire  $F_2$  population of 525 broom-corn plants

Phenotype	Observed number	Calculated number	Deviation
Double dwarf.....	22	32 8	10 8
Whisk dwarf.....	114	98 4	15 6
Western dwarf.....	96	98 4	2 4
Standard.....	293	295 4	2 4

Of the 44  $F_3$  progenies from standard phenotypes in  $F_2$ , 3 were pure standard, 6 segregated into approximately 3 standards to 1 whisk dwarf, 18 segregated into standards and western dwarfs, and 17 produced standard, western dwarf, whisk dwarf, and double dwarf plants. There was no noticeable relation between the height of the  $F_2$  standard plants and their genotypic constitution, as determined by growing  $F_3$  generations from them.

## DISCUSSION

From the above results it is apparent that both western dwarf (Acme) and whisk dwarf (Japanese Dwarf) types of broomcorn differ from standard (Evergreen) broomcorn by single height factors. The height factor present in western dwarf is not the same as the one in whisk dwarf broomcorn. For convenience the height factors concerned may be considered as  $AAdd$  in western dwarf,  $aaDD$  in whisk dwarf, and  $AADD$  in the standard type. The  $F_1$  cross of the two dwarfs gives a plant of the constitution  $AaDd$ . The factors  $A$  and  $D$  are completely dominant, as the  $F_1$  plant  $AaDd$  is as tall as a plant of  $AADD$  constitution. In the  $F_2$  generation the following factorial combinations are obtained:

4 $AaDd$	2 $Aadd$	2 $aaDd$	$\frac{1}{2} aadd$
			1 double dwarf
2 $AaDD$	$\frac{1}{3} AAdd$	$\frac{1}{3} aaDD$	
	3 western dwarf	3 whisk dwarf	
2 $AADd$			
$\frac{1}{9} AADD$			
9 standard			

Examples of each of the above genotypes were obtained in the 112 strains in the  $F_3$  generation in 1930.

Because of the lack of information regarding the origin or introduction of the whisk dwarf and western dwarf types, it is probable that both originated as mutations from some standard variety by the loss of a single but different height factor. The whisk dwarf appeared on farms about 1860, and the western dwarf type some 20 years later.

The dwarf broomcorns have a parallel in corn dwarfs,<sup>4,5</sup> with the exception that broomcorn reproduces normally even in the double recessive condition. Also, there is no apparent reduction in the quantity of seed produced by the presence of either recessive dwarf factor in broomcorn. (Fig. 2.) Based on the corn analogy,<sup>6</sup> standard broomcorn would be considered as the normal type.

<sup>4</sup> EMERSON, R. A., and EMERSON, S. H. GENETIC INTERRELATIONS OF TWO ANDROMONECIOUS TYPES OF MAIZE, DWARF AND ANTER EAR. *Genetics* 7 203-236, illus 1922

<sup>5</sup> KEMPTON, J. H. INHERITANCE OF DWARFING IN MAIZE. *Jour Agr Research* 25 297-321, illus 1923

<sup>6</sup> EMERSON, R. A., and EMERSON, S. H. Op cit (See footnote 4)

Standard broomcorn attains a height of from 9 to 12 feet under favorable conditions. The  $F_1$  plants of crosses between dwarf broomcorn and any other sorghum are about this same height, and thus suggest a simple explanation for at least a part of the "hybrid vigor" obtained in the  $F_1$  crosses. Other sorghums apparently usually carry the missing  $D$  or other complementary height factors, the result in crossing dwarf broomcorn with other sorghums being an  $F_1$  plant the height of standard broomcorn and taller than most of the common sorghums. The  $F_1$  plants of crosses between different sorghum groups usually are tall and exhibit hybrid vigor. The two height factors here described, however, do not account for other manifestations of hybrid vigor in sorghums, such as late maturity, thick culms, and an increased number of nodes.

#### SUMMARY

Broomcorn is divided into varietal and commercial classes mainly on the basis of relative height.

In crossing a standard (tall) broomcorn with western dwarf or whisk dwarf broomcorn, an  $F_1$  plant the height of the standard parent is obtained. A single-factor segregation of three tall to one dwarf is obtained in the  $F_2$  generation.

A cross of western dwarf and whisk dwarf broomcorns gave an  $F_1$  plant the height of standard broomcorn. The  $F_2$  generation gave a 2-factor segregation of 9 standard to 3 western dwarf to 3 whisk dwarf to 1 double dwarf.

Considering standard broomcorn as possessing two height factors,  $A$  and  $D$ , the western dwarf lacking the  $D$  factor, and whisk dwarf lacking the  $A$  factor, a simple explanation is apparent for the standard height of the  $F_1$  cross between these two dwarfs and for the tall  $F_1$  plants obtained when either type of dwarf broomcorn is crossed with other sorghums. The tall  $F_1$  plants usually are considered entirely a result of hybrid vigor.

# DEHISCENCE OF THE BOLL OF LINUM RIGIDUM AND RELATED SPECIES<sup>1</sup>

By A C DILLMAN, Associate Agronomist, and J C BRINSMADE, JR., Assistant Agronomist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture<sup>2</sup>

## INTRODUCTION

The purpose of this paper is to describe the mechanism of a very unusual method of dehiscence of the boll or capsule in the yellow-flowered flax, *Linum rigidum* Pursh, and in several related species native to the Great Plains area of North America extending from Canada to Mexico. The dehiscence of *L. rigidum* is entirely different from that of other species of the genus *Linum* and of dehiscent fruits of other plants.

In the common cultivated flax, *Linum usitatissimum* L., the bolls are either semidehiscent, that is, they open at the apex and crack slightly along the margins of the segments, as in the fiber and seed flaxes commonly grown in Europe and the United States, or they are indehiscent, as in most varieties of Argentina and India.<sup>3</sup> In another distinct type, *L. usitatissimum crepitans* Bonningh, the bolls are widely dehiscent so that the seeds fall as soon as the bolls are ripe. Several regional strains of this dehiscent flax have been described recently by Elladi,<sup>4</sup> a Russian investigator. This variety is grown to a limited extent in the Ukraine and is found also in Portugal, Spain, Austria, Germany, and eastern Russia. Dehiscence occurs also in *L. angustifolium* Huds., which Tammes<sup>5</sup> suggests may be the wild prototype of our cultivated flax, as the two species hybridize readily and produce fully fertile seeds. Prompt dehiscence of the ripe bolls also occurs in most wild species, including *L. perenne* L. of Europe and *L. leursii* Pursh, the somewhat similar perennial flax, which is native to the Great Plains and Rocky Mountain region of North America.

In the several species mentioned, dehiscence is due simply to dehydration and shrinkage of certain tissues, or to the greater or unequal shrinkage of certain parts of the boll. The semidehiscent bolls of common flax, and even the fully dehiscent bolls of the variety *crepitans*, will close if wet by rain or dew, and open again as they dry out.

## DEHISCENCE IN LINUM RIGIDUM

In the species *Linum rigidum* and the several evidently related species the mechanism of dehiscence, if it properly can be called dehiscence, is very different from the species described above. In *L. rigi-*

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<sup>2</sup> The writers desire to acknowledge the generous cooperation of F. H. Hillman, botanist, Division of Seed Investigations, Bureau of Plant Industry, who made the drawings shown in fig. 2, and of Merritt N. Pope, agronomist, Division of Cereal Crops and Diseases, who made the section of the boll of *Linum rigidum* shown in fig. 3.

<sup>3</sup> DILLMAN, A. C. DEHISCENCE OF THE FLAX BOLL. Jour. Amer. Soc. Agron. 21: 832-833, illus. 1929.

<sup>4</sup> ELLADI, E. V. FLAX WITH DEHISCENT CAPSULES. Trudy Prikl. Bot. i Selek. (Bul. Appl. Bot. and Plant Breeding) 22: 455-471, illus. 1929. [In Russian. English summary by C. Elladi, p. 470-471.]

<sup>5</sup> TAMMES, T. THE GENETICS OF THE GENUS LINUM. Bibliographia Genetica 4: 1-36. 1928.

*dum* the ripe bolls remain tightly closed however dry they may become, but open wide when wet by rain or dew. This mechanism has not been described heretofore, so far as the writers have been able to learn. It was first observed in July, 1926, at Mandan, N. Dak., where the species is widely distributed on the native short-grass sod. In examining some plants one morning when they were wet with dew it was seen that the bolls were wide open, whereas they had been closed the previous evening. Later in the day some dry plants were dipped in water and the bolls opened at once.



FIGURE 1—A, Plant of *Linum berlandieri* showing the mature bolls in dry condition. B, A panicle branch and detached bolls (a), which were dipped in water just before the photograph was taken, showing the appearance of the bolls as they open when wet. Plants collected near San Antonio, Tex., May 24, 1931.

A photograph of a plant of *Linum berlandieri* Hook., a species very similar to *L. rigidum*, collected near San Antonio, Tex., is shown in Figure 1. The appearance of the mature bolls as they open when wet can be seen on the panicle branch (B) and in the single bolls (a), which were dipped in water just before the photograph was taken. The immature bolls, with sepals still attached, did not open.

## THE ORGAN OF DEHISCENCE

The organ of dehiscence in *Linum rigidum* is shown in Figure 2. The ovary of the flax flower consists of five carpels which form the five segments of the mature boll or capsule. In *L. rigidum* the five carpels separate in the mature boll (fig. 2, A, *a*), each carpel having two seeds inclosed in the membranous sac (endocarp), which has openings at the apex through which the seeds may escape. (Fig. 2, C, *a*.) The two seeds in each carpel are separated by a septum attached to the outer wall. Each segment of the boll is attached to the receptacle by a hingelike organ which consists of two distinct tissues. The outer tissue, which functions as a hinge, is wax yellow, translucent, tough, pliable, and nonabsorbent. (Fig. 2 A, *b*; B, *b*.) The inner tissue consists of four or five rows of colorless hygroscopic motor cells which absorb free water very rapidly. (Fig. 2, C, *b*.) If a small drop of water is placed on the outer tissue at the point *b* it

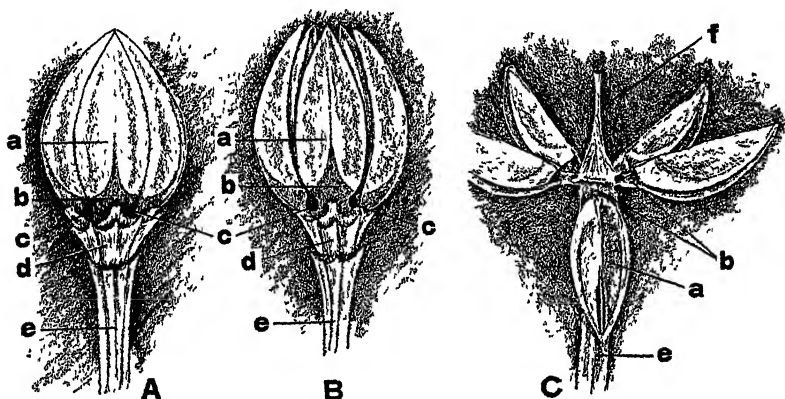


FIGURE 2—A, Mature boll of *Linum rigidum* in dry condition; B, the same boll beginning to open a few seconds after a drop of water had been placed at the orifice *c*; C, the boll fully open. The five segments (*a*) of the boll are separated and each is attached at its base to the receptacle by a hingelike organ, *b*. The minute orifice (*c*) allows water to enter the boll, where it is rapidly absorbed by the colorless tissue C, *b*, which swells and pushes outward the separate segments, thus opening the boll. The fluted pedicel (*e*) bears a sort of capital (*d*), which in overripe plants is finally separated by abscission from the remainder of the pedicel. The growing seeds (ovules) appear to be attached by a short placental thread to the apex of the central placental column shown in C, *b*.

will not be absorbed. If, however, the drop of water is moved to the margin of the orifice (fig. 2, A, *c*), it will be absorbed rapidly by the inner tissue of the organ and in about 20 seconds the boll will begin to open. (Fig. 2, B.) In about a minute the boll will be completely open as in Figure 2, C.

This mechanism suggests the mechanical principle of the thermograph in which the differential expansion of two metals due to temperature changes is made use of. In this organ of the flax boll the differential expansion, due to absorption of water by the inner tissue and nonabsorption by the waxlike outer tissue, pushes outward the separate segments, thus opening the boll. A small drop of water, or a film of water in the form of dew, is sufficient to open the boll. The boll will open completely in about 1 minute after wetting, and will close again as soon as the inner tissue dries out, usually in 5 to 10 minutes in a dry warm room.

This remarkable organ is not injured by use or incapacitated by reasonable age. The bolls of one plant in the possession of the senior writer have opened and closed probably a hundred times by wetting and diving during a period of two years. The bolls of specimens 20 to 50 years old in the National Herbarium at Washington opened readily when a drop of water was placed on them. A specimen of *Linum multicaule* Hook. collected in 1846 (now 85 years old) reacted weakly to warm water.

Photomicrographs of a section of the boll of *Linum rigidum* are shown in Figure 3. In A, the section shows one segment of the boll with the inclosed seed (a), the motor organs of two segments (b), the

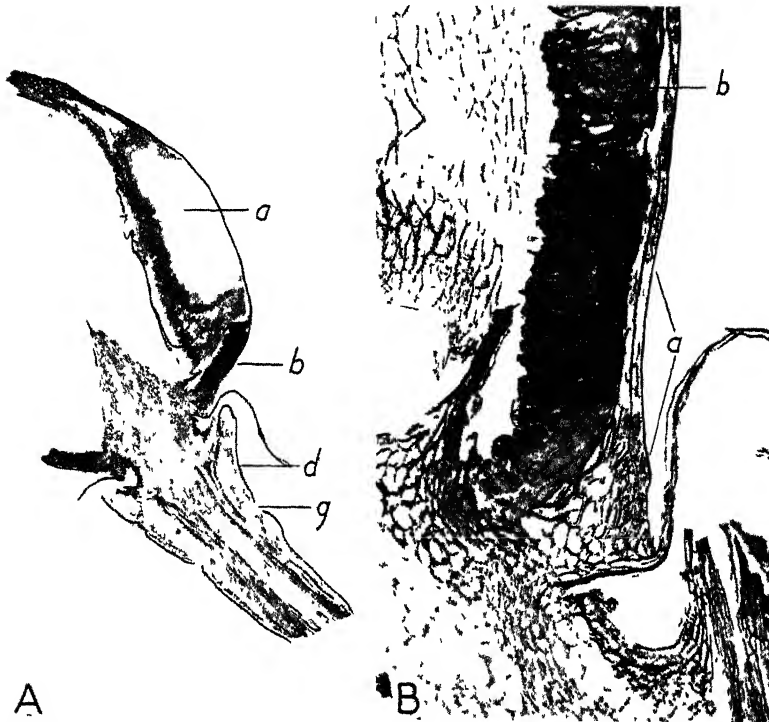


FIGURE 3—A, Vertical section of the boll and pedicel of *Linum rigidum*, showing outline of a seed, a, the motor organ, b, the upper capitallike portion of the pedicel, d, and the point of abscission, g.  $\times 12$ . B, Motor organ, shown in detail. The lignified cells, a, form a straplike hinge to which the mass of water-absorptive motor cells, b, are attached.  $\times 72$ .

capitallike portion of the pedicel (d), and the area of abscission (g). It is apparent that abscission is not due to the formation of special cork cells, as occurs in many plants, but is simply the separation of certain cells in a definite area of the cortex of the pedicel. In B, the motor organ is shown in more detail. It consists of a supporting tissue (a) of thick-walled cells which form the straplike hinge, and a mass (b) of colorless thin-walled motor cells. By the use of a microscope these motor cells can be seen to expand instantly when a film of water is added to a thin dry section of the organ. The section shown in this figure was stained with two dyes, the motor

cells staining dark violet with haematoxylin and the straplike tissue staining red with safranin.

#### SHEDDING OF THE BOLL

This mechanism of dehiscence appears to be a remarkable provision of nature to assure the distribution of the seed on wet soil under conditions favorable for germination. In practice, however, it is not highly effective. The seeds may be retained for a considerable time within the sacs, but finally emerge through the openings at the tip of the segments (Fig 2, C, *a'*). The mere opening of the boll after wetting is not sufficient to insure shedding of the seeds. The application of some force, as a beating rain or a high wind, seems to be required to cause the seed to be shed. Moreover, the seeds are slow to germinate, a considerable period of wet weather, or more likely a freezing temperature, is required before the seeds will grow. As a matter of fact, the bolls may be shed from the plant before the seeds escape.

The shedding of the boll is brought about by abscission tissue which cuts off the upper portion of the short pedicel in the form of a sort of capital (fig 2, A, *d*), to which the boll is attached. The fluted pedicel (*e*) with its capital is suggestive of a miniature Corinthian column. In overripe plants this part (*d*) of the pedicel separates at the line of abscission (the dark line shown in the drawing) from the remainder of the pedicel. The bolls also are sometimes broken off at the receptacle, the place of attachment to the pedicel, instead of at the usual line of abscission.

#### SPECIES OF LINUM DEHISCENT WHEN WET

In the classification of the family Linaceae, Small<sup>6</sup> in his key to the genus *Cathartolimum* distinguishes a group of species as follows. "Sepals deciduous, capsules with cartilaginous thickenings at the base. VII. *Rigida*." No further mention is made of these "cartilaginous thickenings" in the detailed descriptions of the species listed. This is the only reference that the writers have found to this organ of dehiscence in *Linum rigidum* and related species.

The absence of sepals is characteristic of the ripe bolls. The deciduous sepals are broken off by the first opening of the mature bolls, which occurs when they are wetted by dew or rain. Sepals are present on the green and immature bolls.

The senior writer has examined all species of *Linum* and *Cathartolimum* found in the National Herbarium and has observed that this organ of dehiscence is present only in the species listed by Small in his groups *Rigida* and *Multicaulia*. In a few immature specimens it could not be determined whether the mechanism was present, although very likely it is characteristic of all species in the group *Rigida*. It was definitely present and operative, when a drop of water was applied to the bolls, in one or more specimens of the species listed in Table 1.

<sup>6</sup> SMALL, J. K. LINACEAE. In North American Flora 25 (pt. 1) 67-87. New York 1907.

TABLE 1 —*Species of Cathartolimum in the National Herbarium in which the bolls were found to be dehiscent when wet*

National Herbarium specimen No	Species	Locality	Year collected
691099	<i>Cathartolimum puberulum</i> .....	New Mexico.....	1912
891067	do.....	do.....	1916
13717	<i>C. vernale</i> .....	Mexico boundary survey.....	
739639	do.....	New Mexico.....	1902
358825	<i>C. alatum</i> .....		
13713	<i>C. australe</i> .....	Chihuahua, Mexico.....	1899
265733	<i>C. rigidum</i> .....	New Mexico.....	1869
13709	do.....	Kansas.....	1893
504824	do.....	Oklahoma.....	1891
1004457	<i>C. berlandieri</i> .....	Texas.....	1914
13691	<i>C. aristatum</i> .....	Puebla, Mexico.....	1907
589482	<i>C. multicaule</i> .....	Texas.....	1846
	do.....	Oklahoma.....	1913

As stated above, the organ was not found in any species except in Group VII, *Rigida*, and the one species *Cathartolimum multicaule* in Group VIII, *Multicaulia*. It is probable that this species properly belongs in the group *Rigida*.

This organ of dehiscence is so distinctive that it might very well be used as a generic character to distinguish the group of species in which it is present. It is very evident that these species are closely related, and, so far as known, they are native to the Great Plains and adjacent areas in North America. The character of dehiscence in these species is just opposite to that of other species of *Linum* and of the dehiscent pods of other plants. The bolls of these species open by wetting, the others by drying. Possibly this distinctive organ and its operation should be defined by new terms.

In Britton's Manual<sup>7</sup> *Linum rigidum* is described as "perennial (?)" At Mandan, N. Dak., *L. rigidum* is either an annual or a winter annual. Numerous mature plants have been marked from time to time, but none of them has lived over winter. Seeds have been sown in the fall, but germination has not been observed except in the fall of 1930, when a long period of wet weather brought about germination. It is probable that many plants are winter annuals, the seedlings overwintering under the protection of prairie grass and snow.

#### SUMMARY

This paper describes a peculiar plant organ that occurs in the bolls or capsules of the yellow-flowered flax, *Linum rigidum* Pursh, and in those of several related species native to the Great Plains of North America. The capsules of these species open when wet by rain, in contrast to those of other flaxes and the pods of legumes, which dehisce by drying. The opening of the capsule in *L. rigidum* is dependent on the definite action of a hingelike organ which forms the attachment of each segment of the capsule to the receptacle. The five segments of the boll are pushed open by the rapid expansion of the inner tissues of the organ, which absorb water through minute orifices at the base of and between the segments. So far as the

<sup>7</sup> BRITTON, N. L. MANUAL OF THE FLORA OF THE NORTHERN STATES AND CANADA. 1080 p. New York 1901.

writers are aware, this organ of plant movement has not heretofore been described.

It is suggested that new terms be used to distinguish this organ and its action from the dehiscence of dry capsules. It is believed, also, that a separate genus might properly be made of the group of related species now included in the genus *Cathartolinum* of Small, which are distinguished by this peculiar organ of the capsule. The organ probably occurs in all of the 14 species listed by Small under his groups *Rigida* and *Multicaulia*.



# EFFECT OF TEMPERATURE ON RATE OF DECAY OF SUGAR BEETS BY STRAINS OF PHOMA BETAE<sup>1</sup>

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## INTRODUCTION

Tompkins and Nuckols<sup>4</sup> noted recently the importance of *Phoma betae* (Oud.) Frank as a wound parasite of stored sugar beets. No scientific data are available concerning the effect of environmental conditions that might conceivably favor or retard penetration of the exposed tissues of the sugar-beet crown by the fungus. During the years 1926 to 1929 the writers made detailed ecological observations at commercial storage piles in northern Utah. Accumulated evidence soon indicated that temperature probably constitutes one of the most important of the environmental factors associated with crown rot of the sugar beet in storage.

This paper reports the results of studies on the relative rate of decay of sugar beets brought about by four isolations of *Phoma betae* under controlled temperature conditions and on the relation of temperature and storage period to the quantitative effects of each of these four isolations as decay producers.

## STRAINS OF PHOMA BETAE USED

Four isolations of *Phoma betae* were employed in the experiments herein reported. The sources of this material are shown in Table 1. All the pure cultures were originally obtained by making monospore cultures, following which Brown's method<sup>5</sup> was used in order to insure freedom from bacterial contamination. The isolations were then thoroughly tested for pathogenicity to mature beets by inoculating freshly exposed crown tissues.

TABLE 1.—*Source of material of Phoma betae used for inoculation*

Culture No	Source (Logan, Utah)	Date of original isolation
252	Field	Oct. 18, 1927
259	Storage pile	Nov. 30, 1927
260	do.	Do
261	do.	Do

<sup>1</sup> Received for publication July 14, 1931, issued February, 1932.

<sup>2</sup> Resigned Jan. 15, 1930.

<sup>3</sup> Resigned Nov. 30, 1929.

<sup>4</sup> TOMPKINS, C. M., and NUCKOLS, S. B. DEVELOPMENT OF STORAGE DISEASES IN SUGAR BEETS RESULTING FROM WOUND INJURY. *Phytopathology* 18: 939-941, illus. 1928.

— and NUCKOLS, S. B. THE RELATION OF TYPE OF TOPPING TO STORAGE LOSSES IN SUGAR BEETS. *Phytopathology* 20: 621-635, illus. 1930.

<sup>5</sup> BROWN, W. TWO MYCOLOGICAL METHODS. I. A SIMPLE METHOD OF FREEING FUNGAL CULTURES FROM BACTERIA. II. A METHOD OF ISOLATING SINGLE STRAINS OF FUNGI BY CUTTING OUT A HYPHAL TIP. *Ann. Bot. [London]* 38: 401-404, 1924.

The size of spores was determined only for isolations 252 and 259, since isolations 260 and 261 did not readily form mature pycnidia on prune agar except with extreme age. Pycnidia selected from 20-day-old cultures were crushed in glycerin. One hundred spores from each of the two isolations were measured with a filar micrometer. The mean length and the mean width of the spores from both isolations are given in Table 2.

TABLE 2—Measurements of spores from two 20-day-old cultures of *Phoma betae*

Strain No	Length	Width
252.....	$5.595 \pm 0.047$	$3.815 \pm 0.028$
259.....	$4.756 \pm .073$	$3.349 \pm .036$
Difference.....	$.839 \pm .087$	$.466 \pm .046$

The spores of isolation 252 are significantly longer and wider than those of isolation 259, as shown by the differences  $0.839 \pm 0.087$  and  $0.466 \pm 0.046$ , respectively. Because of (1) this significant difference in spore size, (2) distinct differences between the various isolations observed in culture, and (3) the difference in aggressiveness shown in attacking sugar-beet tissue, discussed later in the paper, these four isolations are considered as representing four strains of *Phoma betae*.

#### METHODS USED IN DETERMINING RATE OF DECAY

Since the technic used in the experiments varied, details are given for each experiment in chronological order. In all experiments sound, healthy beets were used. In order to expose fresh tissue free from cork cells all crown tissues above the base of the lowest leaf scar were removed prior to inoculation by means of a steel knife that had been dipped in 95 per cent alcohol and flamed. Either whole beets or sections of beets were inoculated as indicated below. The inoculum, comprising vigorous growing colonies of the four strains of the fungus, was prepared in Petri dishes containing approximately 20 c. c. of freshly prepared prune agar. The age of the inoculum varied slightly in each of the experiments. The inoculum with agar substratum was added in each case so as to cover the exposed surface of the crown. The inoculum was thus in direct contact with the cut surface of the beet tissue, and the agar served as a protective agency against desiccation. As a further precaution against premature drying of the inoculum, a piece of sterile absorbent cotton moistened with sterile water was placed on top of the inoculum and held there by means of adhesive tape. Unless otherwise indicated, inoculated beets were rolled up in glassine bags to prevent drying of the host and fungus.

Details for each of the four experiments are as follows:

##### Experiment 1

Storage period, January 10 to February 20, 1929, 41 days.

Age of inoculum, 15 days.

Purpose of experiment:

(1) To determine the effect of temperature on the organism and the host.

(2) To determine rate of decay production for each fungus strain.

Beets, after inoculation with the various strains, were placed at the four different temperatures in the control chambers, as shown in Table 3.

At the completion of the storage period, the weights of the whole beet, of diseased tissue, and of the remaining healthy tissue were taken.

*Experiment 2*

Storage period, February 20 to April 1, 1929, 40 days.

Age of inoculum, 17 days.

Purpose of experiment Same as in experiment 1.

In order to avoid as a possible source of error the variability in morphology and chemical composition of individual beets, one-fourth sections of beets were used instead of whole beets. The crown tissues were removed as in experiment 1, and the beets were then quartered longitudinally.

Procedure for determining the effect of (1) temperature and (2) pathogenicity of strains:

(1) The four one-fourth sections of the same individual beet were inoculated with the same fungus strain, after which each one of these quarters was subjected to a different temperature in order to determine the effect of temperature on the fungus strain and the host

(2) In a second test each one of the four sections of an individual beet was inoculated with a different strain of the fungus and subjected to similar controlled storage conditions in order to determine more accurately the relative pathogenicity of each organism

At the conclusion of the experiment observations were made on the weight of both diseased and healthy tissue.

*Experiment 3*

Storage period, April 4 to May 8, 1929, 34 days.

Age of inoculum, 20 days.

Purpose of experiment: Same as in experiments 1 and 2 and, in addition, to test methods of procedure with primary reference to moisture exchange.

The two procedures were used as indicated in experiment 2.

The inoculated beet sections were rolled in glassine bags.

The data included the weight of the beet sections before and after storage and the weight of the diseased tissue.

*Experiment 4*

Storage periods:

November 26 to December 8, 1929, 12 days.

November 26 to December 20, 1929, 24 days.

November 26, 1929, to January 2, 1930, 37 days.

November 26, 1929, to January 13, 1930, 48 days.

Age of inoculum, 20 days.

Purpose of experiment: Same as in experiments 1, 2, and 3, but conducted under better controlled conditions of moisture

Instead of using the one-fourth sections of the entire section of a beet, as in the two preceding experiments, 10 comparable pieces whose weight was approximately 13 gm each were cut from  $\frac{1}{2}$ -inch-thick sections of large beets, as shown in Figure 1. This was done in order to obtain pieces of similar anatomical and chemical composition and for a more accurate control of the tissue variability within the beet. The tissue near the lateral grooves was especially avoided because of its different composition and character.

The two procedures explained under experiment 2 were employed.

The inoculated sections were placed on a raised glass platform in a covered glass moisture chamber. Moistened filter paper supplied the necessary humidity. The moisture chambers were then placed in the different control chambers at the desired temperature.

The number of inoculations made in each of the experiments 1, 2, and 3 is given in Table 3.

In experiment 4, four lots of beet sections, of 48 sections each, were inoculated with the four fungus strains 252, 259, 260, and 261, respectively. Each lot of inoculated sections was then subdivided into four lots of 12 sections each, which were held in the control chambers at 10° C. for 12, 24, 36, and 48 days, respectively.

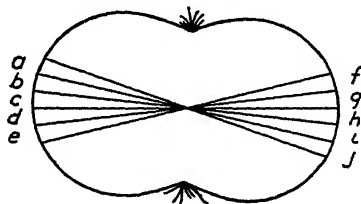


FIGURE 1.—Cross section of large sugar beet, showing method used in experiment 4 of cutting samples (a to j) from comparable regions

TABLE 3—Number of whole beets or sections of beets inoculated with various strains of *Phoma betae* and placed in control chambers at various temperatures for experiments 1, 2, and 3

Strain No	Inoculated beets or sections <sup>a</sup> held at indicated temperature (° C ) in experiments 1, 2, and 3												Total beets used
	1°			5°			10°			15°			
	1	2	3	1	2	3	1	2	3	1	2	3	
252.....	4	20	24	4	20	24	4	20	24	10	20	24	198
259.....	4	20	24	4	20	24	4	20	24	10	20	24	198
260.....	4	20	24	4	20	24	4	20	24	10	20	24	198
261.....	10	20	24	10	20	24	12	20	24	6	20	24	214

<sup>a</sup> Individual beets were inoculated in experiment 1, in later experiments, sections of beets were used

The controlled temperatures used in these experiments were made possible by placing temperature-control chambers in cold-storage rooms of a commercial ice and storage company at Salt Lake City, Utah. Each of the temperature-control chambers had a capacity of 5,832 cubic inches and was well insulated. Each chamber had a separate temperature-control device, which consisted of an accurate thermoregulator and a magnetic switch for making and breaking the heating-element circuit. The temperatures were controlled to within  $\pm 0.3^{\circ}\text{C}$ . Air temperatures in these control chambers were adjusted to  $1^{\circ}$ ,  $5^{\circ}$ ,  $10^{\circ}$ , and  $15^{\circ}$ , the range approximating rather closely actual field-storage conditions.

Student's methods<sup>6</sup> were used for most of the statistical calculations and the probabilities given as values of  $P$ .

The probable errors of the mean for the dimensions of the spores were calculated by Bessel's formula, and the probable errors of the differences were calculated by the usual formula,  $\pm \sqrt{(E_1)^2 + (E_2)^2}$ .

## RESULTS OF EXPERIMENTS

Since the pathogenicity of the four strains of *Phoma betae* had been carefully tested shortly after the original cultures were isolated, it was deemed sufficient to make reisolutions from diseased beets or sections of beets only in experiments 2 and 3 to serve as checks. The reisolutions were made on prune-agar plates by incubating thereon small pieces of beet tissue, part of which gave distinct evidence of infection, although all were cut under aseptic conditions. The strains of the organism were again recovered in pure culture, as indicated in Table 4.

As the results of the first experiment were not used in the primary calculation, it was deemed advisable to present them separately. Table 5 gives the weight in grams of diseased tissue for all the beets inoculated in experiment 1. The results are grouped according to the strain used for inoculation and the temperature at which the inoculated beets were stored. These results indicate differences in the rate of decay caused by the different strains and also strong differential response to the various temperatures.

<sup>6</sup> FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. 230 p., illus. Edinburgh and London, 1925.

TABLE 4—*Reisolations from parts of beets which had been inoculated with various strains of Phoma betae and held at various temperatures in experiments 2 and 3*

Experiment No and storage temperature (° C )	Reisolations			
	Total	Negative	Positive	
	Number	Number	Number	Per cent
Experiment 2				
1°	51	1	50	98
5°	52	1	51	98
10°	55	8	47	85
15°	62	19	43	69
Experiment 3				
1°	64	3	61	95
5°	66	5	61	92
10°	73	16	57	78
15°	68	8	60	88

TABLE 5—*Effects of inoculating sugar beets with various strains of Phoma betae and storing for 41 days under different temperatures in experiment 1*

## STRAIN 252

Beet No	Weight of total, healthy, and diseased tissue of inoculated beets stored at temperature (° C ) indicated											
	1°			5°			10°			15°		
	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased
1	Gms 285	Gms 260	Gms 25	Gms 360	Gms 310	Gms 50	Gms 370	Gms 70	Gms 413	Gms 368	Gms 45	Gms 45
2	445	420	25	465	430	35	190	140	68	0	68	68
3	315	275	40	310	280	30	285	270	15	345	325	20
4	470	450	20	411	390	21	225	183	40	122	72	50
5										132	90	42
6										355	325	30
7										300	185	115
8										211	150	61
9										235	210	25
10										160	150	10
Total	1,515	1,405	110	1,546	1,410	136	1,070	895	175	2,341	1,875	466
Average	378 75	351 25	27 50	386 50	352 50	34	267 50	223 75	43 75	234 10	187 50	46 60
Diseased			P ct 7 23			P ct 8 80			P ct 16 36			P ct 19 91

## STRAIN 259

1	405	370	Gms 35	185	150	Gms 35	150	90	Gms 60	120	0	Gms 120
2	173	165	8	275	250	25	180	90	90	170	50	120
3	275	230	45	170	140	30	120	25	95	54	40	14
4	380	330	50	190	160	30	287	200	87	88	40	48
5										155	110	45
6										350	190	160
7										120	50	70
8										60	25	35
9										50	10	40
10										135	60	75
Total	1,233	1,095	138	820	700	120	737	405	332	1,302	575	727
Average	308 25	273 75	34 50	205	175	30	184 25	101 25	83	130 20	57 50	72 70
Diseased			P ct 11 19			P ct 14 63			P ct 45 05			P ct 55 84

TABLE 5—Effects of inoculating sugar beets with various strains of *Phoma betae* and storing for 41 days under different temperatures in experiment 1—Contd

## STRAIN 260

Beet No	Weight of total, healthy, and diseased tissue of inoculated beets stored at temperature (° C ) indicated											
	1°			5°			10°			15°		
	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased
	Gms	Gms	Gms	Gms	Gms	Gms	Gms	Gms	Gms	Gms	Gms	Gms
1.....	260	240	20	370	350	20	232	220	12	402	330	72
2.....	355	330	25	385	360	25	515	470	45	360	260	100
3.....	228	225	3	417	410	7	145	125	20	60	35	25
4.....	278	270	8	240	230	10	162	140	22	155	145	10
5.....										115	70	45
6.....										210	140	70
7.....										170	120	50
8.....										110	15	95
9.....										70	60	10
10.....										68	60	8
Total.....	1,121	1,065	56	1,412	1,350	62	1,054	955	99	1,720	1,235	485
Average..	280 25	266 25	14	353	337 50	15 50	263 50	238 75	24 80	172	123 50	48 50
Diseased..			P ct 5 00			P ct 4 39			P ct 9 39			P ct 28 20

## STRAIN 261

			Gms			Gms			Gms			Gms
1.....	170	160	10	600	580	20	470	440	30	235	200	35
2.....	175	165	10	710	680	30	285	250	35	345	300	45
3.....	385	370	15	165	160	5	330	300	30	245	185	60
4.....	115	110	5	125	120	5	277	220	57	270	160	110
5.....	132	110	22	200	180	20	515	480	35	210	170	40
6.....	465	430	35	215	190	25	338	310	28	270	230	40
7.....	248	240	8	270	230	40	530	500	30			
8.....	179	170	9	214	210	4	580	530	50			
9.....	345	330	15	178	170	8	335	310	25			
10.....	285	260	25	315	290	25	415	330	85			
11.....							480	460	20			
12.....							245	210	35			
Total.....	2,499	2,345	154	2,992	2,810	182	4,800	4,340	460	1,575	1,245	330
Average..	249 90	234 50	15 40	299 20	281	18 20	400	361 67	38 33	262 50	207 50	55
Diseased..			P ct 6 16			P ct 6 08			P ct 9 58			P ct 20 95

In experiment 1 whole beets were used for each inoculation. Because of the great irregularity in the weights of diseased tissue from different beets when subjected to the same organisms and temperature, only sections of beet tissue (fig. 1) were used for the later experiments, thus eliminating differences due to variability among individual beets. While this procedure reduced the irregularities in the results, statistical methods showed other variabilities which it is believed might have been due to faulty technic in procuring a uniform quantity of inoculum throughout the tests or to variability in conditions of storage.

In experiments 2 and 3 strains 252 and 259 were found to have the highest relative rate of decay and strain 260 the lowest rate of decay, as shown in the following comparison of average weights of diseased tissue per day for each strain of *Phoma betae*.

Strain No	Diseased tissue (gm)
252.....	0 4958
259.....	. 4968
260.....	2242
261.....	4070

The following probability values were calculated<sup>7</sup> for differences in decay produced by each pair of strains of *Phoma betae* at all temperatures (1°, 5°, 10°, and 15° C) used in experiments 2 and 3

Pairs of strains	Probability value (P)
252 and 259.....	0. 45
252 and 260.....	. 01
252 and 261.....	. 18
259 and 260.....	. 01
259 and 261.....	. 15
260 and 261.....	. 01

These data indicate that strain 260 was the least aggressive and that each one of the three others was significantly different from it. The differences in respect to aggressiveness between strains 252, 259, and 261 are not clearly significant. When the data for these organisms are analyzed for the individual temperatures, as shown in Table 6, the differences are more clearly apparent. Strain 252 was not significantly different from 259 or 261 in amount of decay produced, but was significantly different from 260. Strain 259 was not significantly different from 261, but was markedly different from 260. The amount of decay produced by strains 260 and 261 was significantly different at all temperatures investigated.

TABLE 6.—Probability values for the differences in decay produced by each pair of strains of *Phoma betae* used in experiments 2 and 3

Pairs of strains	Temperature (° C)	Probability value (P) <sup>a</sup>		Pairs of strains	Temperature (° C)	Probability value (P) <sup>a</sup>	
		Experiment 2	Experiment 3			Experiment 2	Experiment 3
252 and 259.....	1	0. 59	0 84	259 and 260.....	1	0 06	0 01
Do.....	5	. 70	. 29	Do.....	5	. 04	. 01
Do.....	10	. 55	. 38	Do.....	10	. 01	. 01
Do.....	15	. 61	. 11	Do.....	15	. 06	. 01
252 and 260.....	1	. 01	. 01	259 and 261.....	1	. 54	. 04
Do.....	5	. 05	. 01	Do.....	5	. 44	. 19
Do.....	10	. 02	. 01	Do.....	10	. 02	. 27
Do.....	15	. 10	. 16	Do.....	15	. 04	. 18
252 and 261.....	1	. 36	. 04	260 and 261.....	1	. 01	. 01
Do.....	5	. 69	. 02	Do.....	5	. 01	. 01
Do.....	10	. 03	. 13	Do.....	10	. 02	. 01
Do.....	15	. 18	. 82	Do.....	15	. 03	. 03

<sup>a</sup> A probability value of 0.05 for *P* was considered the limit of significance

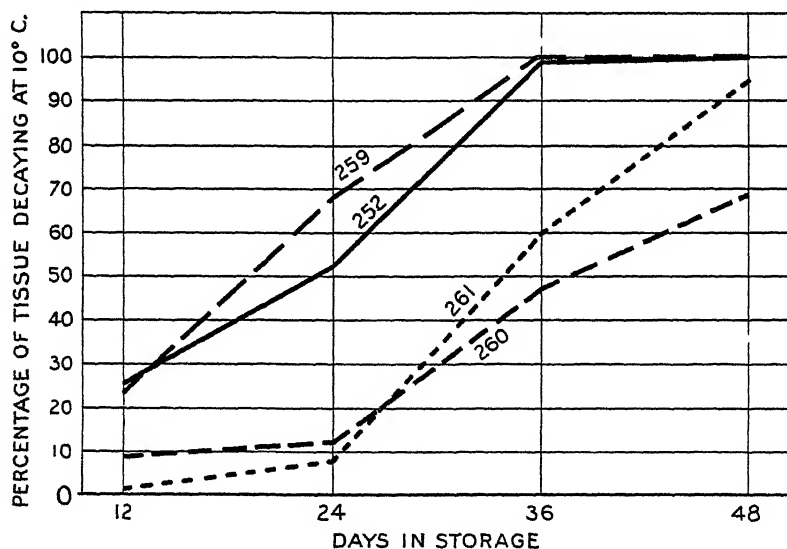
Data showing the effect of temperature on the amount of tissue destroyed by each strain are given in Table 7. These results indicate that the weight of tissue destroyed at 5° C was in no case significantly different from that destroyed at 1°. Strain 261 did not destroy significantly more tissue at 10° than at 1° or 5°. In all other cases, except strain 260 at 5° to 10°, the weights of tissue destroyed by each strain were significantly different for each 5 degrees change of temperature.

<sup>7</sup> A probability value of 0.05 for *P* was considered the limit of significance

TABLE 7—Probability values for differences in weight of tissue destroyed by each strain of *Phoma betae* at temperatures of 1°, 5°, 10°, and 15° C in experiments 2 and 3

Temperature (° C )	Strain No	Probability value (P) <sup>a</sup>	Temperature (° C )	Strain No	Probability value (P) <sup>a</sup>
1° and 5°	252	0.12	5° and 10°	252	0.04
Do.	259	.53	Do.	259	.02
Do.	260	.18	Do.	260	.07
Do.	261	.51	Do.	261	.15
1° and 10°	252	.01	5° and 15°	252	.02
Do.	259	.01	Do.	259	.01
Do.	260	.04	Do.	260	.01
Do.	261	.16	Do.	261	.01
1° and 15°	252	.01	10° and 15°	252	.04
Do.	259	.01	Do.	259	.02
Do.	260	.01	Do.	260	.01
Do.	261	.01	Do.	261	.01

<sup>a</sup> A probability value of 0.05 for *P* was considered the limit of significance

FIGURE 2—Relative progress of decay in each of the four strains of *Phoma betae*

The rate of decay, based on the average of results obtained in experiment 4, is expressed as curves in Figure 2 for strains 252, 259, 260, and 261, respectively. The curve of strain 252 is similar to that of strain 259, whereas the curve of strain 260 resembles that of strain 261. These curves confirm the statistical results presented in Tables 5, 6, and 7, and also show that strains 252 and 259 destroy sugar-beet tissue more rapidly than do strains 260 and 261. Strains 252 and 259 destroyed practically all the available tissue within 36 days, but strains 260 and 261 did not. Strain 260 was again shown to be the least aggressive. Strain 261 attacks slowly, as does 260, but progresses at a rapid rate, approaching the curves of strains 252 and 259 at about the forty-eighth day. These curves, together with the fact that the experiments deal with the relative rate of decay produced by the strains during long storage periods, show why it was not possible to establish significant differences between strains 261 and 252 or between

strains 261 and 259 The curves indicate a probable difference in aggressiveness for strain 261 and show the advantage of running such tests at varied temperatures and for various lengths of storage periods

#### DISCUSSION

*Phoma betae* is undoubtedly the most important wound parasite found in stored sugar beets in the Western States, particularly in northern Utah While no figures are available as to the economic losses resulting from the action of this fungus, it is safe to assume that the damage incurred from year to year has generally reached significant proportions Reduction in the total extractable sucrose, occasioned by rotting of the healthy tissues, and in the weight of the stored beets constitutes the main source of loss

The experimental results herein reported show that temperature is one of the most important ecological factors associated with decay of beets in storage In general, low temperatures retard the penetration of the fungus into exposed crown tissues and the higher temperatures favor it It would therefore seem advisable to delay until the advent of cooler weather the piling of beets in those localities where high temperatures prevail The length of the storage period may determine in large measure whether or not losses will become significant, according to the rate of decay produced by the strains of *Phoma betae* present in the storage pile Although only four strains of the fungus were used in the experiments reported in this paper, the senior writer has isolated from various sources more than 20 strains that gave evidence of morphologic variation Since marked differences in size of spores were found in two of the strains used in these experiments and since similar variations in size of spores have been noted in other strains, there is reason to believe that a number of distinct strains exist The results of the present investigation indicate wide differences in the capacity of these strains to decay sugar-beet tissue

#### SUMMARY

The relation of temperature and length of storage to rotting of sugar beets by the fungus *Phoma betae* (Oud.) Frank has been investigated Distinct morphologic differences have been found between two of the strains used

Four strains of the fungus were studied under controlled conditions to determine their relative capacity for producing decay The data indicate that the four strains of *Phoma betae* tested vary in the rate at which they destroy beet tissue, the differences amounting to as much as 50 per cent but varying according to the temperature and period of storage

Increase in temperature during storage favored increased metabolism of the fungus, with attendant increase in rotting of tissues In general, significant differences were noted for each change of 5° C. in temperature.

Strains 252 and 259 were found to possess similar aggressiveness, although they differed significantly in morphology Both differed markedly from strain 260 in the amount of decay produced Strains 260 and 261, the morphology of which was not studied, were found to be significantly different in their ability to produce decay. It is therefore believed that distinct strains of *Phoma betae* exist



# ROOT CONSTRICTION OF COTTON PLANTS IN THE SAN JOAQUIN VALLEY OF CALIFORNIA<sup>1</sup>

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## INTRODUCTION

Cotton plants may be severely stunted, or even killed, when their roots are choked in hard soil. The injury occurs a little below the surface of the ground, where the soil becomes dry and hardens around the small seedling taproot, thus preventing further expansion. As the plant grows the stalk bulges out over the hard soil and forms an enlarged calloused base, contrasting with the threadlike taproot firmly encased in the hard soil. The plant reaches a stage of development where such constriction of the root causes it to wilt rather suddenly and it will die if the condition is not relieved in a short time.

In Texas, instances of root constriction have been reported in which cotton plants growing in heavy clay soils were strangled as a result of root constriction when the soil was compacted by continuous rain or excessive irrigation<sup>2</sup>. A similar effect was observed by the writer at the United States cotton field station, Shafter, Calif., in 1930. In this case the constriction was caused by withholding water early in the season, thus allowing the soil to become dry and hard near the surface.

The disorder as noted in Texas was recorded as a new disease and named "root strangulation"; but since it is neither physiological nor parasitic, it is hardly to be reckoned as a disease unless the word is used in its broadest sense, to include any departure from normal structure or function. Cook,<sup>3</sup> in discussing leaf cut, or tomosis, a common disorder of cotton seedlings, recognizes a class of ecological disorders intermediate between physiological diseases and mechanical injuries or traumatisms. To this intermediate class the strangulation of plants by root constriction may be added, though the causal factors are purely mechanical.

## OBSERVATIONS AND STUDIES

At Shafter, Calif., several dead plants were found in plots of cotton that showed no general indications of stress conditions. When examined the roots of these plants were found to be severely constricted just under the surface mulch where they entered the firmer soil. In most cases the root just above the constriction was enlarged beyond the normal size at the base of the stalk, and calloused. The reduction from these large calloused bases to the constricted root below was usually very abrupt. Often a reduction from a stem 1.5 to 2 cm. in diameter to a root about 1 mm. in diameter would occur in the space of 1 cm. or less.

<sup>1</sup> Received for publication July 29, 1931, issued February, 1932.

<sup>2</sup> ANONYMOUS. [COTTON PLANTS ARE STRANGLED TO DEATH BY HARD, DRY CLAY IN A NEW DISEASE OF COTTON. (Item) Science (n. s.) 71 (1947): XIV. 1930.

<sup>3</sup> COOK, O. F. LEAF-CUT, OR TOMOSIS, A DISORDER OF COTTON SEEDLINGS. U. S. Dept. Agr., Bur. Plant Indus. Circ. 120. 29-34. 1913.

The constrictions of the roots were usually from 3 to 6 inches or more in length, extending through the extremely hard dry soil near the surface. Beneath this hard dry soil layer the roots appeared normal in every respect. In many cases there were no lateral roots near the surface and the plants were merely resting on flat, calloused bases. In such cases the plants were kept erect largely by the tension of the threadlike taproots that held these large calloused bases firmly against the hard soil. When the plots were irrigated many such plants fell prostrate as the soil softened and gave way under the bases.

The first observations of the disorder at Shafter were recorded July 2, 1930, the condition appearing in a series of plots that were planted April 21. These plots had been irrigated on April 15 by flooding and were harrowed lightly before planting. On June 13 the stand was thinned to about 12 inches between plants, and this operation was followed by a shallow cultivation, leaving a surface mulch from 2 to 3 inches deep. The average plants in the plots were about 12 to 15 inches high and had been flowering for several days when the first dead ones were observed. The dead and severely wilted plants were slightly smaller than the average. Further investigations showed that most of the plants were more or less constricted, and many of the smaller ones were observed to show slight symptoms of a deficient water supply. In the following days more of these plants wilted and died rather suddenly, and the condition became so severe that the plots were irrigated on July 8. Several plants that were severely wilted immediately before irrigation were tagged for further study. Some of these recovered very slowly from their wilted condition, while others regained turgidity soon after irrigation but remained a dull bluish color for several days, indicating water stress. Only a few plants failed to recover, and these were practically dead when irrigated.

Several of the tagged plants were removed July 10, two days after irrigation, by digging them carefully and washing the soil from the roots. All of these plants had badly constricted taproots and very few old lateral roots near the surface, but they had many white rootlets springing from the bases of the stalks and taproots. These rootlets ranged in length from very short stubs to more than one-fourth inch, as shown in Figure 1.

Additional specimens of the plants that were severely wilted before irrigation were removed July 22. Most of these recovered completely, and in every case in which the top of the plant had recovered the taproot was filled out to normal size. A few of the tagged plants were left undisturbed throughout the season. These showed no ill effect of the early constriction but developed normally and produced good crops of cotton.

Figure 2 shows a plant severely wilted from root constriction, in comparison with an adjacent normal plant. When the plot was irrigated a few minutes after this photograph was taken the wilted plant fell because the soil softened beneath its base. Other plants in the same plot that were wilted to about the same extent immediately before irrigation made recoveries, some apparently complete, others only partial. The degree of wilt represented by the wilted plant in Figure 2 appeared to be about the limit of stress from which a plant could recover to normal.



FIGURE 1 —Parts of constricted cotton plants two days after irrigation, showing the development of white rootlets and the quick response of the plants to irrigation (Natural size)

## PHOTOGRAPHIC RECORDS

In a further study of this disorder, in another set of plots, two series of natural-size photographs were made of root-constricted plants, showing portions of the taproots before and after irrigation. The first series was begun July 23 in a plot of cotton that was planted May 24, and the second series was begun August 15 in a plot planted June 7. Neither of these plots had been irrigated after planting, previous to the beginning of the studies. A group of three plants in each plot was used for study. The first group will be referred to as series 1 and the second group as series 2. Photographs are shown of only one plant in each group. In series 1 the photographs of plant



FIGURE 2—Constricted cotton plant (at right) showing severe condition of wilt, in comparison with an adjacent normal plant. This severely wilted condition is reached in a few hours after wilting starts. Such plants do not recover at night.

1 typify all three plants studied. In series 2 only one of the three plants survived.

## SERIES 1

The upper portions of the taproots of the three plants in series 1 were photographed July 23. This was done by digging a trench about 8 to 10 inches deep close to the plants and washing away the soil from one side of the taproots by means of a small pressure tank and hose. Natural-size photographs were taken as soon as the taproots were clearly exposed. The soil was then carefully replaced about the roots and the plot irrigated.

The roots of these plants were again photographed in a similar manner on August 8 for comparison with the photographs taken on July 23. Figure 3 shows the condition of the taproot of plant 1

of this series at the time of the first and second exposures. These observations merely confirm the result of the more casual determinations already made showing that the plants were able to fill out the

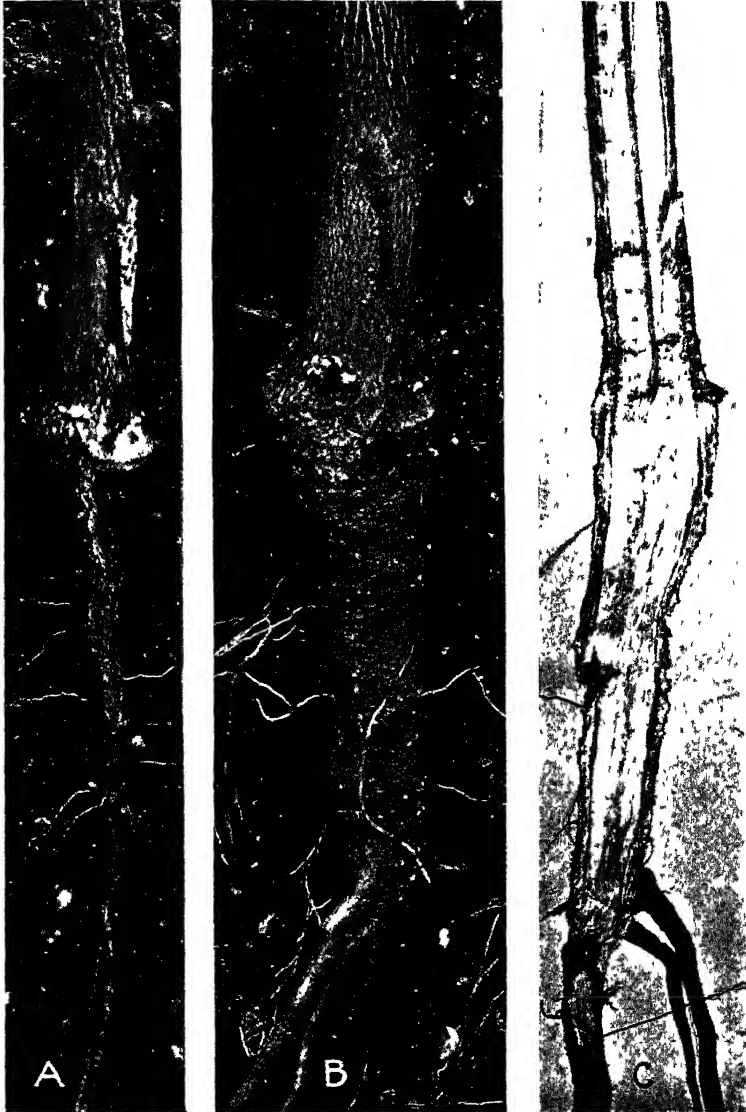


FIGURE 3—Three views of base of stalk and taproot of plant 1, series 1. A, Severely constricted taproot as it appeared before irrigation, July 23, B, taproot showing recovery from constriction, August 8, 16 days after irrigation, C, longitudinal section of the root after recovery (Natural size)

constrictions of the roots in a very short time after irrigation. Each of the three plants under observation showed severely constricted roots before irrigation and a complete recovery 16 days after irriga-

tion The plants were removed from the field on August 8, and the taproots were split in longitudinal sections to show the newly developed wood (Fig 3, C) No lines of demarcation were visible between the old wood of the constricted root and the new wood formed after irrigation

#### SERIES 2

In series 2, consisting of three plants, the roots were first exposed for photographing on August 15, primarily to determine the rate of recovery of the constricted roots of plants that recovered promptly aboveground. These plants were in a plot that was planted June 7 and received no irrigation after seeding Many plants in this plot were dead or dying from root constriction when the first exposure was made. Figure 4 shows a section of a row near the location of the three plants of this series, taken just before irrigation, showing plants in several stages of wilt caused by root constriction This plot was



FIGURE 4—Section of row near the location of the three plants selected for study in series 2 This photograph, taken just before the first irrigation after planting, shows several plants severely wilted from root constriction, together with normal-appearing plants

irrigated August 15 immediately after the first set of photographs was taken.

Plant 1 of this series was turgid and green when photographed, but wilted soon afterward and did not make a complete recovery Plants 2 and 3 were slightly wilted at 9 a. m., before the roots were disturbed Plant 2 failed to recover and was dropped with plant 1 from this investigation, leaving only plant 3 to be studied for the rate of root expansion. This plant recovered from wilting promptly and assumed a normal appearance aboveground a few days after irrigation A second exposure was made of this plant on August 21 and a third exposure on August 26 Figure 5 shows the condition of the taproot at the time of the first, second, and third exposures, respectively. Some idea of the rapid development that takes place in constricted roots after irrigation may be had by comparing these views The rapid development of lateral roots near the surface of the ground appears remarkable.

In Figure 5, A, which shows the plant before irrigation, it may be seen that there were no lateral roots near the surface at the time of the first exposure. In B, which was taken six days after the first exposure and irrigation, many small white lateral roots are shown These roots were very tender and some were broken, others were exposed entire, but care was taken not to disturb them more than was

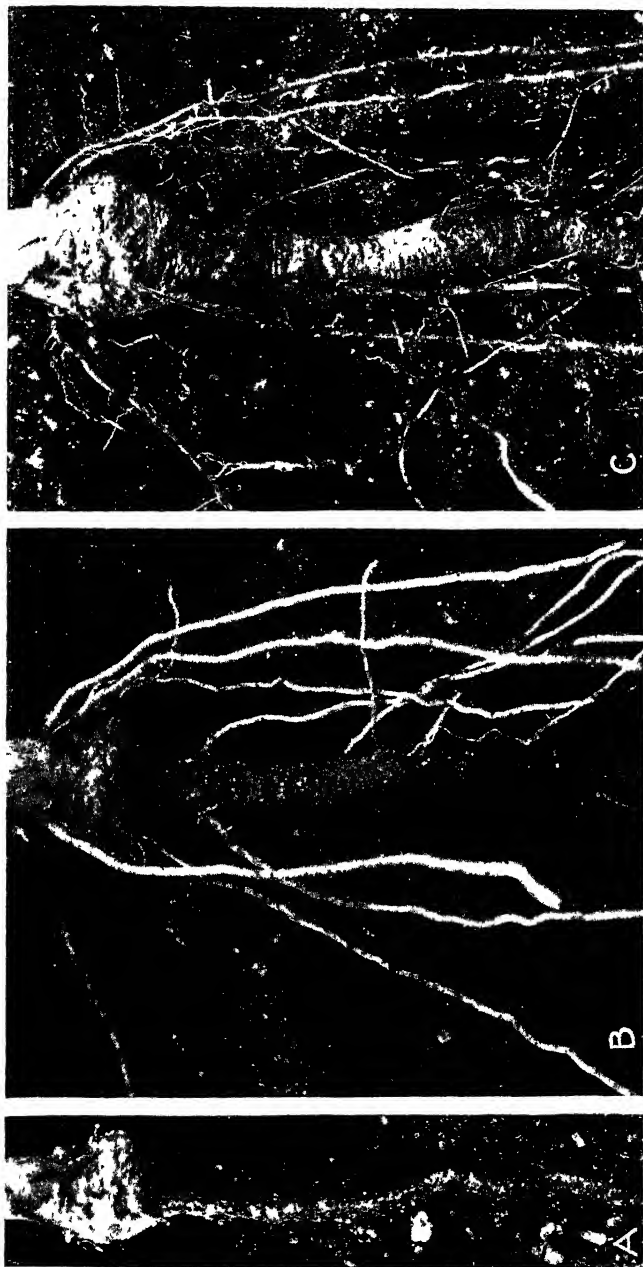


FIGURE 5.—Base of stalk and roots of plant 3, series 2, showing rapid development after irrigation, (about seven-eighths natural size) A, View August 15, before irrigation, showing taproot severely constricted with no lateral roots near the surface, B, view August 21, six days after irrigation, showing taproot greatly increased in diameter, and development of many small lateral roots, some of them more than 6 inches long, C, view August 26, five days after B and 11 days after A, showing taproot practically normal in size and lateral roots of tough woody structure with numerous secondary branches

necessary, since further study of the plant was desirable. Figure 5, C, which was taken five days after B and 11 days after A, shows these laterals to be of tough, woody structure, with many small secondary branches

Several neighboring plants were removed from the plot August 21 and cross sections were made of the taproots through the constriction. A small hard center of old wood was clearly distinguishable in these sections, with a large ring of clear, semitransparent wood and rather heavy bark. The proportions of old wood, new wood, and bark on an average root were, respectively, three thirty-seconds, three thirty-seconds, and one-sixteenth of an inch. The new wood was very soft, and the greater part of it could be scraped off easily with the thumb nail into a clear jellylike mass. Similar cross sections of roots removed August 26 showed no definite lines of demarcation between the old and new wood, but the cambium was very active and a thin outer portion of the wood was rather soft.

### DISCUSSION

Constriction of taproots was found to be general at the United States cotton field station in plots that were not irrigated for a long time after planting, and in many cases it became necessary to irrigate before the plants had reached a stage of development where irrigation appeared desirable, except as a measure to prevent some losses in seedling stand. Some plots at the station were irrigated early in the season during the seedling stage of the plants, and the roots of these plants developed normally, while the roots of plants in adjoining plots that were not irrigated became constricted. The general practice among cotton growers in the San Joaquin Valley is to irrigate freely rather early in the season, in order to develop a large plant, and then to stop irrigation in an effort to force maturity of the crop. While this practice is not considered a good cultural method, it prevents root constriction and is probably the reason why the disorder has not been reported heretofore. The objection to irrigating early in the season before the cotton has reached the flowering stage is that the plants may grow too rank and fail to mature as large a crop of bolls as they would otherwise. Moreover, the plants that have too much water at first may develop shallow root systems and are therefore likely to suffer in dry weather, so that both the quality of the fiber and the yield may be impaired. It appears that root constriction may interfere somewhat with the application of improved cultural methods on the sandy soils that become very hard when dry; but since the injury is not permanent and does not appear to affect the later development of the plants in any way, it is probably of minor importance.

### SUMMARY

Cotton plants grown at the United States cotton field station at Shafter, Calif., in 1930 were observed to wilt and die suddenly as a result of taproot constriction. The soil in which these plants were grown is a light sandy loam that becomes very hard when dry, and the constricted plants were found in each case in plots that had not been irrigated after planting.

Irrigation corrected the condition that caused constriction, and the plants that were not too severely injured recovered after irrigation and developed into normal, well-fruited plants.

Photographs of constricted roots taken before and after irrigation show that the plants made a rapid recovery from the disorder as soon as the cause was removed.

In one series of photographs the development of new lateral roots near the surface of the ground is shown. A plant having no surface lateral roots before irrigation is shown on the sixth day after irrigation with numerous small, white lateral roots, some of them more than 6 inches long. Eleven days after irrigation these new laterals had greatly increased in length, were tough and woody, and had many branches.

In cross sections of constricted roots made during the rapid growth after irrigation a large ring of soft semitransparent wood tissue was observed between the old wood and the bark. Six days after irrigation this new wood tissue could be scraped off with little effort into a clear, jellylike mass, but 11 days after irrigation no lines of demarcation were perceptible to the naked eye between the new and the old wood.

The disorder is probably of little importance to the cotton grower under present conditions, but it may interfere with the utilization of the best cultural methods unless some practical method other than irrigation is devised to correct the conditions causing it.



# HETEROTHALLISM AND HYBRIDIZATION IN *TILLETIA TRITICI* AND *T. LEVIS*<sup>1</sup>

By H H FLOR

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## INTRODUCTION

The most destructive disease of wheat in the Pacific Northwest is bunt or stinking smut. Seed treatment is not entirely effective on fall-sown wheat because of soil infestation. Consequently, the most promising means of control appears to be the development and use of resistant varieties.

A number of varieties apparently resistant to bunt have been developed. For a number of years these varieties maintained their resistance, but they are now being attacked with increased severity from year to year. Gaines<sup>2</sup> has shown that these varieties are being attacked by new forms of *Tilletia tritici* (Bjerk.) Wint. and *T. levis* Kuhn. Prior to 1918 only *T. tritici* was known to occur in the State of Washington (10)<sup>3</sup>. By the inoculation of selected varieties with a number of field collections, Gaines in 1928 found that the resistant varieties had maintained their resistance to the old form of *T. tritici*, but that two additional forms of *T. tritici* and four forms of *T. levis* were present in this region.

This increasing amount of smut in previously resistant varieties showed the importance of a thorough consideration of the rôle of physiologic specialization in a breeding program and emphasized the necessity for obtaining fundamental information concerning the origin of these new forms as well as their prevalence and distribution.

The occurrence of physiologic specialization in the cereal smuts has been repeatedly demonstrated. According to Stakman (16), physiologic forms may be differentiated by (1) cultural characters, (2) physicochemical reactions, (3) morphology, and (4) pathogenicity. Pathogenicity as shown by varietal reaction has been the criterion most widely used for differentiation and is of most importance from the standpoint of the plant breeder.

Usually little if any effort has been exercised by the various investigators to insure the purity of the physiologic forms of smut with which they have worked. The usual procedure has been to obtain collections from various localities or from resistant varieties and test these on a number of varietal host testers directly, or after the collection has been increased on a susceptible host. In some instances an attempt has been made to purify the collections by increasing them on their respective differential hosts, i. e., the variety that each collection is able to infect but that is not attacked by other collections.

<sup>1</sup> Received for publication July 16, 1931; issued February, 1932. The investigations herein recorded were conducted by the Division of Cereal Crops and Diseases in cooperation with the Washington and Oregon Agricultural Experiment Stations.

<sup>2</sup> GAINES, E. F. WHY SMUT HAS BEEN INCREASING. U. S. Dept. Agr., Off. Coop. Ext. Work, Ext. Path. 6 (2) 14-15. February, 1928. [Mimeographed.]

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 58.

Obviously, this method would strain out those forms that were unable to infect this variety. However, it would give no assurance that the collection had been reduced to a single physiologic form, as it is possible that a number of forms or hybrid forms exist that are able to attack any given variety. In order to study the importance of mutation and hybridization in the development of pathogenically different physiologic forms, it is imperative that the work be started with lines as pure as possible.

The present investigation was undertaken to study the pathogenicity of single and paired monosporidial cultures of *Tilletia tritici* and *T. levis* and thus learn as much as possible concerning the nature of infection, the importance of hybridization in the development of new forms, and the possibility of developing improved methods for the purification of the physiologic forms.

#### HISTORICAL REVIEW

The fusion of sporidia in certain smuts has been observed by a number of investigators. Kniep (11) in 1919, working with *Ustilago violacea* Pers., was the first to show that this fusion occurs only between certain sporidia and that therefore the smut is heterothallic. Since then a number of species of the Ustilagineae have been studied and found to be heterothallic, except for Christensen's (2) report of infection by monosporidial lines of *U. zeae* (Beckm.) Ung. Zillig (17) confirmed the work of Kniep and further showed that conjugation occurred between sexual strains of different physiologic forms. Kniep (12) observed conjugation between the sporidia of a number of smooth-spored species of *Ustilago* and also between different echinulate-spored species and reticulate-spored species. He also observed conjugation between sporidia of certain smooth-spored and echinulate-spored species, but observed none between sporidia of reticulate-spored and those of either smooth-spored or echinulate-spored species. Dickinson (5) observed hyphal fusion in the host tissue between monosporidial cultures of *U. hordei* (Pers.) Kell. and Sw. and *U. nuda* (Jens.) Kell. and Sw., but did not permit his plants to grow to maturity. Apparently interspecific fusion is common in the smuts. However, none of these investigators completed their tests. They did not report whether the interspecific crosses were able to cause infection, as shown by the production of mature chlamydospores in the host plant. This information is exceedingly important from the point of view of the investigator interested in the origin and development of physiologic forms. Hanna and Popp (9) found that *U. avenae* (Pers.) Jens. and *U. levis* (Kell. and Sw.) Magn. were heterothallic and that a monosporidial culture of one species mated readily with one of opposite sex of the other species. From this mating a smutted panicle was produced which was somewhat intermediate in appearance between the loose and covered types, and the spores were echinulate.

A number of investigators have attempted to infect wheat plants with cultures of the bunt organism. Sartoris (15) failed to obtain infection, although he grew his wheat in large flasks containing a culture of the organism. Kienholz and Heald (10) likewise failed to obtain infection when the seed was inoculated with cultures derived

from the chlamydospore mass. Bodine and Durrell (1) obtained infection by inoculating wheat seedlings with cultures derived from secondary sporidia produced by a chlamydospore mass culture.

#### FORMATION OF SPORIDIA

The chlamydospores of *Tilletia tritici* and *T. levis* germinate by forming a promycelium into which the protoplasm of the spore passes. As the promycelium elongates, the protoplasm is confined to the upper portion, and septa are laid down as the basal portion is evacuated. At the tip of the promycelium is produced a crown of 8 to 24 long slender sporidia, which in this paper are termed "primary" sporidia. All cultures used in the tests reported in this paper were derived from single primary sporidia. These primary sporidia commonly fuse to form the H-shaped sporidia. Usually both the single primary sporidia and the H-shaped sporidia germinate by sending out a single germ tube or mycelial filament which may or may not produce sickle-shaped secondary sporidia. However, both types of primary sporidia occasionally produce more than one germ tube.

#### MATERIALS AND METHODS

The monosporidial cultures used in these tests were derived from three strains of *Tilletia tritici* and two of *T. levis* that had been collected by E. F. Gaines in the Pacific Northwest and differentiated by their action on wheat varieties.

The methods of making single sporidial isolations as described by Dickinson (4) and Hanna (7) for smuts of the Ustilago type were tried but were not satisfactory. Single spores of *Tilletia tritici* and *T. levis* did not germinate normally on culture media, although in mass normal germination was obtained. Kienholz and Heald (10) found this to be true when they attempted to start cultures with single chlamydospores. Furthermore, the sporidia were produced in a crown at the tip of the promycelium and were difficult to separate. Until fully mature they could not be separated from the tip of the promycelium without injury, and when mature many of them were fused to form the H-shaped sporidia.

Two fairly satisfactory methods were used in isolating single sporidia. In both methods the smut ball was carefully removed from the head, dipped momentarily in 95 per cent alcohol, flamed, and then crushed with forceps over a Petri dish of nonnutrient agar. This was incubated at 15° C. from four to seven days until mature primary sporidia were formed but before there was much germination of the primary sporidia.

In the first method of isolation a suspension was made of the sporidia in sterile water and this suspension streaked with a platinum loop on a thin layer of nonnutrient agar in a Petri dish. The sporidia germinated in from 6 to 24 hours by sending out germ tubes into which the entire protoplasmic contents of the sporidium passed. The sporidia that were sufficiently isolated were then transferred to Thaxter's potato hard agar. This was accomplished with a glass needle drawn out to a diameter equal to two-thirds the length of a primary sporidium and rounded at the tip so as not to cause injury. The needle was sterilized by dipping it in alcohol. The sporidium

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was removed, under the 16-mm objective of a microscope, by touching the needle to the evacuated portion of the sporidium and germ tube, and was deposited on the nutrient agar by drawing the needle across the surface at an oblique angle. The sporidium was invariably deposited at the beginning of the stroke and, if the needle was not forced too deeply into the agar, could be readily seen and the isolation verified by looking through the bottom of the Petri dish with the 16-mm objective.

The second method was somewhat similar to the first in that all the operations were performed under the 16-mm objective. All the sporidia were removed with the glass needle from the tip of promycelium as soon as mature and transferred to a Petri dish of non-nutrient agar. They were then separated from one another with a glass needle and transferred to nutrient agar one at a time.

The subsequent treatment was the same in both methods. The isolated sporidia were incubated from 7 to 10 days at 15° C. At the end of the incubation period the sporidia that were growing had usually developed a colony about 0.1 cm in diameter and were visible to the naked eye. The colony was then transferred to a test tube of potato agar containing 1 per cent dextrose. Only 1 per cent was used because a deficiency of sugar in the medium was found to be conducive to the maintenance of that stage of the fungus characterized by the abundant production of secondary sporidia.

Because of the apparent difficulty in obtaining infection from bunt cultures, the following technic was adopted. Prelude wheat, a susceptible spring variety, was soaked for one hour in a 1-400 Uspulun<sup>4</sup> solution, washed thoroughly, and then dried. The seed was germinated in sterile Petri dishes on filter paper. When the germinating sprouts were one-fourth of an inch long they were inoculated by holding the seed between sterile forceps, puncturing the stem near the base with a sterile needle, and working the inoculum into the wound. The inoculated seedlings were again placed on moist filter paper in sterile Petri dishes and incubated four days at 15° C. They were then transferred to cans of sterile soil, kept in temperature tanks maintained at 15° for approximately two weeks, and then transplanted to a bench of steamed soil in a greenhouse maintained at 15° to 17°.

The single primary sporidial cultures tested were paired in all possible combinations, and 15 seedlings were employed in each test. Lights were used in the greenhouse, and the wheat headed about 45 days after it was transplanted to the bench.

## EXPERIMENTAL RESULTS

### HETEROTHALLISM IN *TILLETIA TRITICI*

The pathogenicity of 12 single primary sporidial cultures derived from the same wheat head of *Tilletia tritici*, form 3, was tested alone and in all possible paired combinations. Although 15 plants were inoculated in each case, that number seldom survived, owing to the severity of the injury at the time of inoculation. As the object of the test was primarily to determine which matings produced infection, as evidenced by smut balls in the head, the number of surviving plants in cases where infection occurred was not important. However, it is

<sup>4</sup> A commercial seed disinfectant.

recognized that the results in cases in which infection did not occur, even when all inoculated plants survived, while strongly indicative, may not have been conclusive

It is possible that pathogenicity is not synonymous with sexual compatibility. It may be influenced by physicochemical or other factors entirely separated from sex. However, until more information concerning the relationship of sexuality and pathogenicity becomes available, it seems advisable to consider the ability of two monosporidial lines to produce smut balls in the wheat head as a measure of their sexual compatibility.

The results obtained in pairing 12 single primary sporidial cultures of form 3 of *Tilletia tritici* are presented in Table 1. These show that the species is heterothallic, for in no case was infection produced by a single culture. A number of sex groups appear to be involved. Hanna (8) and Christensen (2) have shown that this is true in *Ustilago zeae*. Cultures 209 and 213 appeared to belong to one group which when paired with 240, 243, 263, 303, 304, or 306 were able to produce infection. Although 240 and 243 did not cause infection when paired with 213, it is probable, in view of the similarity of reaction of 209 and 213 to the other numbers in this group and because of the small number of plants involved, that they belong to the same group as 263, 303, 304, and 306. Culture 235 produced infection only when mated with 302 and 305, showing the existence of another sexually compatible group. Culture 262 failed to cause infection when paired with any of the other cultures. This indicates either that it had lost its vigor or that another sex group exists.

TABLE 1.—Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of form 3 of *Tilletia tritici*.

Culture No	209	213	235	240	243	262	263	302	303	304	305	306
209.....	—	—	—	+	+	—	+	—	+	+	—	+
213.....	—	—	—	—	—	—	+	—	+	+	—	+
235.....	—	—	—	—	—	—	—	+	—	—	+	—
240.....	+	—	—	—	—	—	—	—	—	—	—	—
243.....	+	—	—	—	—	—	—	—	—	—	—	—
262.....	—	—	—	—	—	—	—	—	—	—	—	—
263.....	+	+	—	—	—	—	—	—	—	—	—	—
302.....	—	—	+	—	—	—	—	—	—	—	—	—
303.....	+	+	—	—	—	—	—	—	—	—	—	—
304.....	+	+	—	—	—	—	—	—	—	—	—	—
305.....	—	—	+	—	—	—	—	—	—	—	—	—
306.....	+	+	—	—	—	—	—	—	—	—	—	—

#### HYBRIDIZATION OF PHYSIOLOGIC FORMS OF *TILLETIA TRITICI*

In Tables 2 and 3 are given the results of pairing monosporidial cultures of forms 1 and 2 of *Tilletia tritici* with two cultures of form 3 which were known to be sexually compatible from preliminary work (6). Although these tests were limited and none of the four sporidia selected from either form 1 or 2 were compatible with one another, they showed that these forms readily hybridized with form 3. Culture 297 of form 1 caused infection when paired with culture 263 of form 3, and cultures 288 and 293 of form 2 reacted similarly with culture 209 of form 3.

TABLE 2—Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 1 and 3 of *Tilletia tritici*

Form and culture No	Form 1				Form 3	
	205	297	299	300	209	263
Form 1						
205.....	—	—	—	—	—	—
297.....	—	—	—	—	—	+
299.....	—	—	—	—	—	—
300.....	—	—	—	—	—	—
Form 3						
209.....	—	—	—	—	—	+
263.....	—	+	—	—	+	—

TABLE 3—Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 2 and 3 of *Tilletia tritici*

Form and culture No	Form 2				Form 3	
	288	290	291	293	209	263
Form 2						
288.....	—	—	—	—	+	—
290.....	—	—	—	—	—	—
291.....	—	—	—	—	—	—
293.....	—	—	—	—	+	—
Form 3						
209.....	+	—	—	+	—	+
263.....	—	—	—	—	+	—

HYBRIDIZATION OF PHYSIOLOGIC FORMS OF *TILLETIA LEVIS*

The tests of the pathogenicity of single and paired monosporidial cultures of *Tilletia levis* were not so extensive as those of *T. tritici*. A single test was made in which four cultures of form 5 and four of form 7 were paired in all possible combinations. The results are given in Table 4.

TABLE 4—Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 5 and 7 of *Tilletia levis*

Form and culture No.	Form 5				Form 7			
	219	245	264	281	223	283	286	308
Form 5								
219.....	—	—	—	—	+	—	+	—
245.....	—	—	—	—	—	—	—	—
264.....	—	—	—	—	—	+	—	—
281.....	—	—	—	—	—	—	—	—
Form 7								
223.....	+	—	—	—	—	—	—	+
283.....	—	—	—	+	—	—	—	—
286.....	+	—	—	—	—	—	—	+
308.....	—	—	—	—	+	—	+	—

This test showed that the infection phenomenon found for *Tilletia tritici* was also true for *T. levis*. The species apparently is hetero-

thallic, as none of the monosporidial cultures alone was able to cause infection. A number of sex groups appear to be involved. None of the cultures of form 5, when paired with one another, caused infection, but they hybridized with sexually compatible cultures of form 7. Four of the eight cultures tested belonged to one sexually compatible group. Cultures 223 and 286 of form 7 produced infection when paired with cultures 308 of form 7 and 219 of form 5. Culture 283 of form 7 caused infection only when mated with culture 281 of form 5, showing the existence of another sexually compatible group. Cultures 245 and 264 did not cause infection when paired with any of the cultures, thus indicating the existence of another sex group.

#### HYBRIDIZATION OF *TILLETIA TRITICI* AND *T. LEVIS*

The question of hybridization of *Tilletia tritici* and *T. levis* in nature has frequently been raised. The writer has examined over 10,000 bunted heads of wheat and has found that the rough-spored and smooth-spored forms rarely occur together in the same smut ball or even in the same head. However, all degrees of reticulation have been observed. Some of the spores were so finely reticulated that they could be distinguished only with difficulty from the nonreticulated species, while others were so coarsely reticulated that they appeared almost spiny. That there is abundant opportunity for hybridization to occur in nature is shown in a survey made by the writer. In 1930, samples of 50 to 100 smutted heads from each field were collected from fields widely separated in Washington, Oregon, and Idaho, and each head was examined for the presence of the smooth-spored and rough-spored species. In Washington 46 out of 65 collections were mixtures of *T. tritici* and *T. levis*; in Oregon 26 out of 45 were mixtures; and in Idaho 9 out of 10 were mixtures.

The results obtained by inoculating seedlings of Prelude wheat with 5 monosporidial cultures of form 3 of *Tilletia tritici*, and with 5 cultures of form 7 of *T. levis*, in all possible paired combinations, are presented in Table 5. These data show that the two species hybridize readily. Of the 10 cultures tested, 8 appear to belong to one sexually compatible group. Culture 308 of *T. levis* and cultures 263, 303, and 306 of *T. tritici* belong to one sex, while cultures 223, 285, and 286 of *T. levis* and 209 of *T. tritici* belong to the other. Although the pairing 285 + 263 did not cause infection, it is probable, in consideration of the limited number of plants used and because of similarity in reaction of these cultures to the others in all the other cases, that other factors than sex were involved. Cultures 283 and 302 did not produce infected plants and may have belonged to a sex group the complement of which was not included in this test.

The spores produced in heads infected by pairing a monosporidial culture of the reticulate-walled *Tilletia tritici* with one of the smooth-walled *T. levis* were identical in appearance with those of the latter. In this test nine interspecific crosses caused infection and the spores were invariably smooth walled and somewhat ellipsoidal in shape.

TABLE 5.—Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of form 3 of *Tilletia tritici* and form 7 of *T. levis*

Inoculum and culture No	T tritici, form 3					T levis, form 7				
	209	263	302	303	306	223	283	285	286	308
T tritici, form 3										
209	—	+	—	+	+	—	—	—	—	+
263	+	—	—	—	—	+	—	—	+	—
302	—	—	—	—	—	—	—	—	—	—
303	+	—	—	—	—	+	—	+	+	—
306	+	—	—	—	—	+	—	+	+	—
T levis, form 7										
223	—	+	—	+	+	—	—	—	—	+
283	—	—	—	—	—	—	—	—	—	—
285	—	—	—	+	+	—	—	—	—	+
286	—	+	—	+	+	—	—	—	—	+
308	+	—	—	—	—	+	—	+	+	—

## DISCUSSION AND CONCLUSIONS

The pathogenicity of 20 cultures of *Tilletia tritici* and 9 cultures of *T. levis*, which had been derived from single primary sporidia, was tested. In this group were three physiologic forms of *T. tritici* and two of *T. levis*. These forms had been purified by varietal straining and were pathogenically distinct. Prelude wheat inoculated with single cultures was not smutted, but the pairing of two sexually compatible cultures caused normal infection.

The problem of the interrelationship of sex and pathogenicity appears to be complicated by the existence of a number of sex groups. For example, cultures A+B may cause infection, as may C+D, but any combination of A or B with C and D will not. The existence of still other sex groups is indicated by cultures that failed to cause infection when paired with A, B, C, or D. The members of the sex groups are specific, for in no instance was a member of one group able to cause infection when paired with one of another group.

Although the sex groups were very distinct, membership within a sex group was not confined to a particular physiologic form or even to the respective species. Into one sexually compatible group fell 8 lines of form 3, 2 of form 2, and 1 of form 1 of *Tilletia tritici*, and 1 of form 5 and 4 of form 7 of *T. levis*. Form 3 of *T. tritici* readily hybridized with forms 1 and 2 of the same species and with form 7 of *T. levis*. Forms 5 and 7 of *T. levis* also hybridized. These were the only combinations of forms tested, but it is probable that hybridization between other combinations may occur and may be an important factor in the origin of new physiologic forms.

In a study of the importance of hybridization and mutation in the origin of pathogenically different physiologic forms of *Tilletia tritici* and *T. levis*, it is essential to obtain as pure a line as possible of the diploid or pathogenic phase.

Cytological studies of *Tilletia tritici* have been made by Rawitscher (14), Paravicini (13), and Dastur (3). These investigators found that a nuclear fusion precedes chlamydospore formation. Therefore, the chlamydospores are hybrids unless the sporidia or monosporidial cultures that unite prior to chlamydospore formation are genetically identical except for sex.

Until the present time the only method that has been used for the purification of collections, in the determination of physiologic forms, is varietal straining. This has been done by inoculating differential varieties on the theory that those forms in the collection to which the particular variety is resistant will be strained out. Although Games's<sup>5</sup> results appear to bear out this theory, this method gives no assurance that a number of physiologic forms to which the variety may be susceptible are not present. Theoretically the sporidia or gametes of a physiologic form should be genetically identical except for sex.

The cultures derived from the single primary sporidia of *Tilletia tritici* and *T. levis* are heterothallic. Consequently these cultures are haploid and functionally gametic. It should be possible to develop pathogenically pure diploid lines by mating haploid cultures of the progeny to each of their parents and in turn mating haploid cultures of the progeny of this mating to each of the original parental cultures. A repetition of this process should develop pure lines with greater certainty than the methods now used for developing pure lines of animals and the higher plants, for instead of having two variable parental gametes, one gamete would be constant.

#### SUMMARY

*Tilletia tritici* and *T. levis* are heterothallic. Wheat seedlings inoculated with individual cultures derived from single primary sporidia remained healthy, but when inoculated with paired cultures of opposite sex they produced smutted heads.

The monosporidial cultures or lines belong to a number of sex groups. The members of each group are specific in their action, as in no instance was a member of one sexually compatible group able to cause infection when paired with a member of another such group.

Wheat seedlings inoculated with a monosporidial culture of *T. tritici* paired with one of *T. levis* of opposite sex were smutted.

Monosporidial cultures of three forms of *T. tritici* and two forms of *T. levis* were found to belong to the same sex group. The spores produced by this species cross were identical in appearance with those of *T. levis*. The epispore wall was smooth, and the spores were somewhat ellipsoidal and slightly angular in shape.

Ample opportunity for hybridization occurs in nature.

By properly pairing the monosporidial cultures it should be possible to develop pathogenically pure lines of *T. tritici* and *T. levis*.

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# THE VITAMIN A, B, C, AND G CONTENT OF SULTANINA (THOMPSON SEEDLESS) AND MALAGA GRAPES AND TWO BRANDS OF COMMERCIAL GRAPE JUICE <sup>1</sup>

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## INTRODUCTION

During recent years a large annual surplus of grapes has been produced in the United States, and the development of new grape products to take care of this increasing supply is at present an important phase of the grape industry. Because of the general interest in a fruit so abundantly grown, it seemed desirable to study the nutritive value of grapes and grape juices more extensively than had previously been done. Raisins have received the attention of several investigators (3, 12),<sup>2</sup> who apparently agree that this product contains very little if any vitamin A, a small amount of vitamin B, and no vitamin C. However, up to the present time very few data have been published on the vitamin content of fresh grapes and their juices, and because of the value of such information the present experiments were planned to determine the amount of vitamins in two varieties of fresh grapes as well as in two brands of commercial grape juice.

Sultanina (Thompson Seedless) and Malaga (*Vitis vinifera*), European varieties, were the grapes selected for study. The Sultanina or Thompson Seedless is a raisin grape constituting about 90 per cent of the total crop of seedless grapes. In this country it is grown principally in California. The Malaga, a table grape, is also used for raisins and, together with the muscat, forms the chief source of the seed or seeded raisins.

In addition to the fresh grapes, two brands of commercial grape juice were analyzed for their vitamin content. The first, designated as commercial juice No. 1, was a mixture of juices approximately one-third from the Flame Tokay and two-thirds from the Zinfandel, European table and juice grapes, classified as *Vitis vinifera*. In the commercial process the juices after extraction from the fresh fruit are filtered and placed in cold storage until needed. Upon removal from cold storage, they are refiltered, sterilized through a machine at a temperature not to exceed 155° F., and bottled. The bottles of juice are then kept in a water bath held at 150° for 45 minutes. Sometimes a slight amount of tartaric acid is found necessary to bring the composition of the product to the standard formula used by the company. No other ingredients are added to the juice.

Commercial juice No. 2 was prepared from Concord grapes (*Vitis labrusca*), an American variety. In preparing this juice the fruit is washed, stemmed, and crushed, heated to about 135° F., and the juice pressed out. The juice is pasteurized and stored in 5-gallon glass

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 69.

carboys for several weeks to a few months to permit settling, it is then siphoned into bottles and again pasteurized. Sugar is generally an added ingredient.

### VITAMIN A

In the present study the vitamin A content of the grapes and grape juices was determined by the Sherman and Munsell method (14). The basal diet was irradiated to supply vitamin D. After the customary depletion period of from four to five weeks, certain groups of rats were fed daily six times per week weighed portions (1, 3, and 5 gm, respectively) of seeded Malaga grapes. Certain other groups received the Sultanina in the same amounts at the same intervals. The commercial juices were given in daily doses of 2, 3, and 5 c c for the same number of days each week. The results are shown in Figure 1 and Table 1.

TABLE 1—Survival of rats receiving various quantities of grape juice as the sole source of vitamin A

Test food	Daily portion, 6 times per week	Rats	Average weight of rats at 4 weeks of age	Average weight of rats at end of fore period	Average time of fore period	Average time of survival after fore period	Average total time of survival after 4 weeks
	C c	Number	Grams	Grams	Days	Days	Days
Commercial grape juice No. 1 <sup>a</sup>	2	6	45.8	95.2	33.6	21.7	55.3
	3	8	45.9	99.9	33.9	23.3	57.1
	5	8	43.4	95.0	33.5	27.2	60.7
	0	7	46.1	99.3	34.3	30.3	64.6
	2	8	56.0	108.2	35.8	23.6	59.4
Commercial grape juice No. 2 <sup>c</sup>	3	8	52.5	101.2	35.6	19.5	55.1
	5	8	50.2	98.9	35.9	23.6	59.5
	0	8	52.9	105.8	35.8	16.3	52.1

<sup>a</sup> A mixture of juices, one-third from the Flame Tokay and two-thirds from the Zinfandel, which are European table and juice grapes classified as *Vitis vinifera*.

<sup>b</sup> 1 rat lived out the full experimental period.

<sup>c</sup> Juice prepared from Concord grapes, *V. labrusca*.

Sherman (12) reported the presence of vitamin A both in grapes and in grape juice, but the variety of grapes was not indicated. He stated that an ounce of grapes contained 16 to 22 units of vitamin, i. e., 0.57 to 0.7 unit per gram. If a unit of vitamin is present in that amount of the test food necessary to produce a gain of 25 gm. in eight weeks, then 1.75 to 1.43 gm. of the fruit were necessary to produce this unit gain. In the present study, in order to obtain a gain of 25 gm. in eight weeks, it was necessary to feed approximately 5 gm. of grapes in the cases of both Sultanina and Malaga. These results indicate that Malaga and Sultanina grapes are less rich in vitamin A than the grapes studied by Sherman. Nevertheless, the growth response induced by these varieties shows the presence of a small but measurable quantity of vitamin A.

Neither of the commercial juices tested showed any indication of the presence of vitamin A. With but one exception the animals died long before the termination of the experiment and showed no better growth than the negative controls. (Table 1.) Upon autopsy the gross anatomical changes due to a deficiency of vitamin A were found to be as severe in the test animals as in the controls.

## VITAMIN B (ANTINEURITIC)

Recorded data furnish but scanty information regarding the vitamin B content either of grapes or of their juices. Osborne and Mendel in 1920 (10) reported that when 10 c c of commercial grape juice was fed daily it was found to contain some "water soluble B" but in amounts insufficient for the normal growth of rats. Sherman (12) reported the occurrence of 7 to 9 units of vitamin B per ounce of grapes. Both of these investigators, however, were considering the vitamin B complex before cognizance had been taken of the antipellagric factor. There appears to be no recent data regarding the vitamin B content of these products.

The vitamin B (antineuritic) tests were carried out according to a method worked out in this laboratory, similar to that outlined by Chase (1). Rats, 28 days old, were placed on diet 107 G, which furnished, with the exception of the antineuritic vitamin, all factors necessary for normal growth and apparent well-being of the animals. This diet had the following composition: Vitamin B-free casein, 18 per cent, starch, 58 per cent, yeast (autoclaved four hours at 20 pounds pressure), 10 per cent, Osborne and Mendel salt mixture, 4 per cent, butterfat, 8 per cent, and cod-liver oil, 2 per cent. All of the animals were kept on this vitamin B-free diet for two weeks, a period of time judged from former observations in this laboratory to be sufficient to deplete the animals of their store of the antineuritic vitamin.

At the end of this depletion period the rats were given weighed or measured portions of the material to be tested. Both varieties of grapes were readily consumed, but a temporary difficulty was expe-

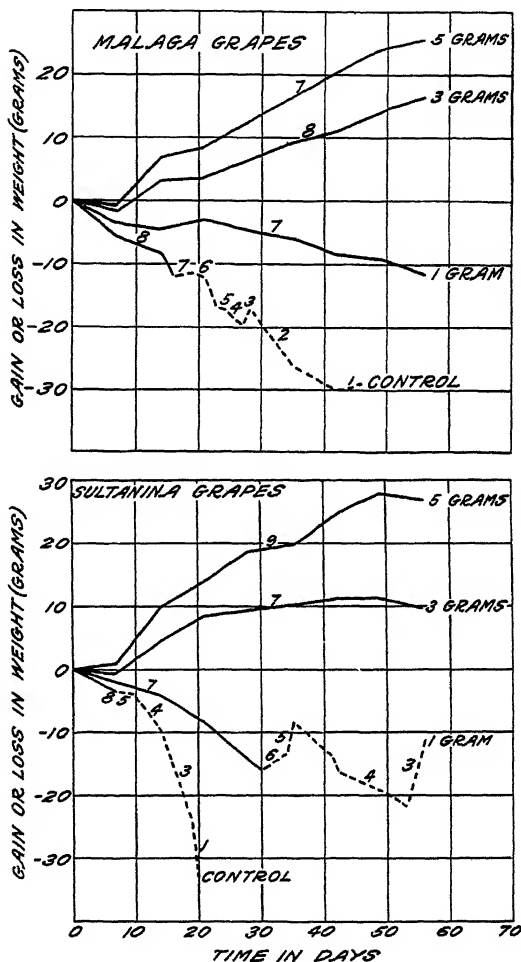


FIGURE 1.—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) as the sole source of vitamin B. The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The weighed quantity of grapes fed daily six times a week is indicated at the end of the curves.

rienced in getting some of the animals to take the grape juice. The Malaga grapes, after the removal of the seeds, were fed in portions of 0.5, 1, 2, 3, 5, and 6 gm, respectively, while the Sultanina grapes were given in amounts of 3, 5, and 6 gm. Each of the two samples of juice were fed daily from small glass containers in 2, 3, and 5 c. c. portions. The results are summarized in Figures 2 and 3.

Figure 2 shows that 5 and 6 gm of fruit in the case of both Malaga and Sultanina grapes induced approximately the same gain in weight,

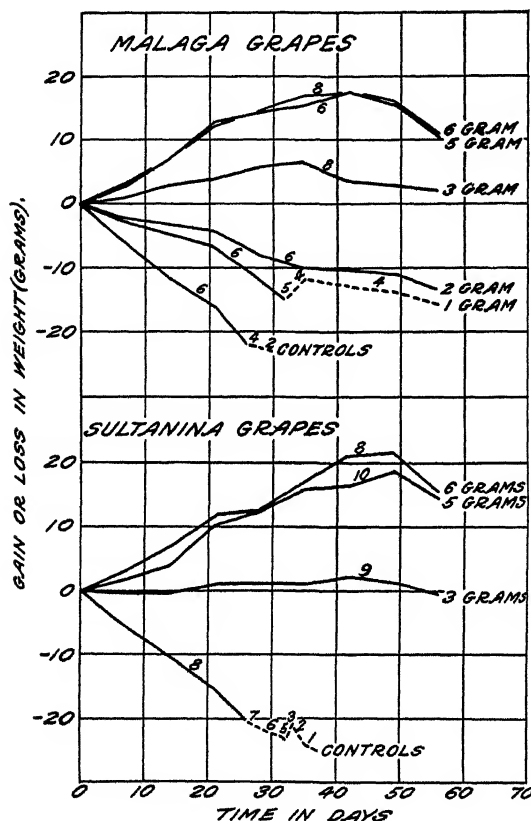


FIGURE 2—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) as the sole source of vitamin B (antiscorbutic). The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The weighed quantity of grapes fed daily six times a week is indicated at the end of the curves.

indicating that the maximum effect of the grapes as a source of vitamin B had been reached. A maximum average gain in weight of 17.5 gm. resulted from feeding both 5 and 6 gm. of Malagas, while 5 gm. of Sultanina produced an average gain of 18.8 gm. and 6 gm. portions of this same grape gave 21.6 gm. gain in weight. These rates of growth of the test animals indicate that the two varieties of grapes are fair sources of vitamin B.

From Figure 2 it may also be observed that the growth curves for these animals show a maximum point between the sixth and seventh week of the test, after which there is a loss in weight to the end of the period. It was difficult to obtain as good grapes at the end of the season as had been fed through the major part of the experiment, but this consistent drop in weight in all of the animals can not

be explained satisfactorily on the basis of poor-quality grapes, since only a comparatively few animals received the inferior product. It is entirely possible that another factor necessary for normal growth was absent from the diet, and upon the depletion of the reserve store of this factor in the animal body the growth curves began to show a decline.

The failure of commercial juice No. 1, in 2, 3, and 5 c. c. daily portions, to induce growth indicates the absence of any measurable quantity of vitamin B in this grape juice (Fig. 3.) On the other hand,

Figure 3 shows that commercial juice No. 2 contains the antineuritic vitamin. The amount is only minimal, however, since a daily portion of 5 c. c. of this juice induced a total gain in weight of only 3 to 4 gm. during the entire test period.

### VITAMIN C

The vitamin C content of grapes and grape juices appears to have been more extensively studied than that of any of the other vitamins. Chick and Rhodes (2) found the juice of grapes to be about one-tenth as rich in the antiscorbutic vitamin as oranges. Givens and Macy (5) found no antiscorbutic properties in dehydrated grape juice which was 14 to 20 months old at the time of testing. According to Merjianian (9), grapes contain vitamin C, the amount varying with the kind of grapes and their freshness. Taking orange juice as 100 for a standard of comparison, Sherman (12) reported that grapes and grape juice have a potency of 4 to 5.

The vitamin C tests on commercial grape juice No. 1 were carried out after the method of Sherman, LaMer, and Campbell (13), the 90-day test period being used. The basal diet

designed as 12 D was a modification of that used by Sherman and had the following composition: Equal parts mixture of bran and oats, 57 per cent; table salt, 1 per cent; butterfat, 9 per cent, heated skim-milk powder, 30 per cent; cod-liver oil, 1 per cent; and yeast, 2 per cent. Six, eight, and ten cubic centimeters of the commercial juice No. 1 were fed from a graduated pipette to groups of guinea pigs every day except Sunday during the test period. Table 2 shows that the survival period of the test animals on this brand of juice was no longer than that of the negative controls and upon autopsy they showed just as severe symptoms of scurvy. Such evidence indicates that commercial grape juice No. 1 contains no vitamin C.

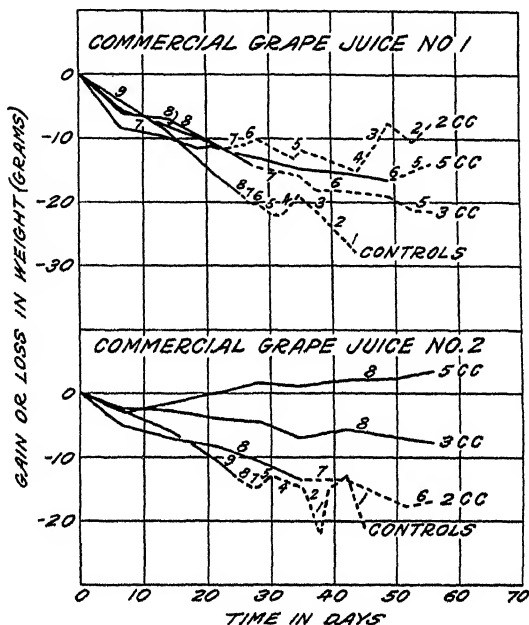


FIGURE 3—Average gain or loss in weight of groups of young rats fed commercial grape juices (No. 1, juice from Flame Tokay and Zinfandel varieties, *Vitis vinifera*, No. 2, juice from Concord grapes, *V. labrusca*) as the sole source of vitamin B (antineuritic). The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The measured quantity of juice fed daily six times a week is indicated at the end of the curves.

TABLE 2—*Survival of guinea pigs receiving various quantities of commercial grape juice No. 1 (juice of Flame Tokay and Zinfandel grapes, Vitis vinifera) as the sole source of vitamin C*

Daily dose, 6 times per week	Guinea pig No.	Weight at beginning	Maximum weight	Weight at end	Survival	Severity of scurvy symptoms at autopsy
		Grams	Grams	Grams	Days	
10 c c -----	245 F	338	371	202	35	Moderate to severe
	254 F	320	316	203	30	Do
	258 F	323	350	232	32	Do
	272 F	327	336	216	29	Moderate
	251 F	322	384	210	34	Do
8 c c -----	252 F	325	335	206	28	Severe
	257 F	323	363	197	32	Do
	261 F	328	337	214	27	Moderate to severe
	274 F	319	319	198	28	Moderate
	259 F	330	401	213	36	Moderate to severe
6 c c -----	263 F	329	349	182	30	Mild
	264 F	324	341	166	33	Severe
	273 F	310	310	242	19	Mild
	162 F	351	427	247	35	Moderate to severe
	262 F	327	347	222	25	Severe
0 c c -----	118 M	344	344	245	27	Moderate
	122 M	404	467	267	35	Severe
	152 M	327	379	203	36	Do
	153 M	300	390	202	37	Do
	170 M	402	459	285	37	Do
	177 M	367	367	221	21	Do
	182 M	361	413	251	31	Do
	259 M	351	423	211	33	Do
	270 M	316	322	217	37	Do

\* The 9 males were controls carried with other experiments in this laboratory

Tests on the commercial juice No. 2 and the fresh grapes were conducted according to the method of Hojer (7, 8), who determines the degree of scurvy by a study of the pathological condition of the teeth. Attention is focused especially on the microscopical examination of a cross section of the root of the incisor tooth of the guinea pig. Höjer claims that this method is more sensitive than the one used by Sherman. A comparative study of these methods has been made by Eddy (4) and Goettch and Key (6). Following Hojer's technique, they found that twice the amount of test food is required to afford complete protection when the criterion depends on a histopathological examination of the teeth, than is apparently necessary when judgment is based on the gross external and internal anatomical changes. Hojer has shown that differential changes in tooth structure begin to take place between the tenth and fourteenth day of the test and are materially sharpened as the experiment continues.

In the present study an 18-day test period was adopted. During this time 10 and 12 c c portions of grape juice were fed to guinea pigs weighing between 300 and 350 gm. Because earlier evidence had indicated that there was little if any vitamin C present in grape juice it was considered unnecessary to feed this test food in smaller quantities. Twelve cubic centimeters was the maximum daily amount which the guinea pigs would take. Seven animals were used. Seeded Malaga grapes were given in 2, 5, 10, and 15 gm. portions to 16 animals, while Sultanina grapes in the same quantities were fed to 12. On the eighteenth day the animals were killed, the incisor teeth removed, and sections prepared.

Figure 4, A, shows the structure of a normal tooth taken from one of the control animals that had received an adequate supply of vitamin C from a daily allowance of cabbage. Figure 4, B, portrays the con-

dition of the tooth of one of the negative control animals fed only the basal diet with no antiscorbutic vitamin for 18 days. From Figure 4, A, it may be observed that the normal tooth possesses a wide band

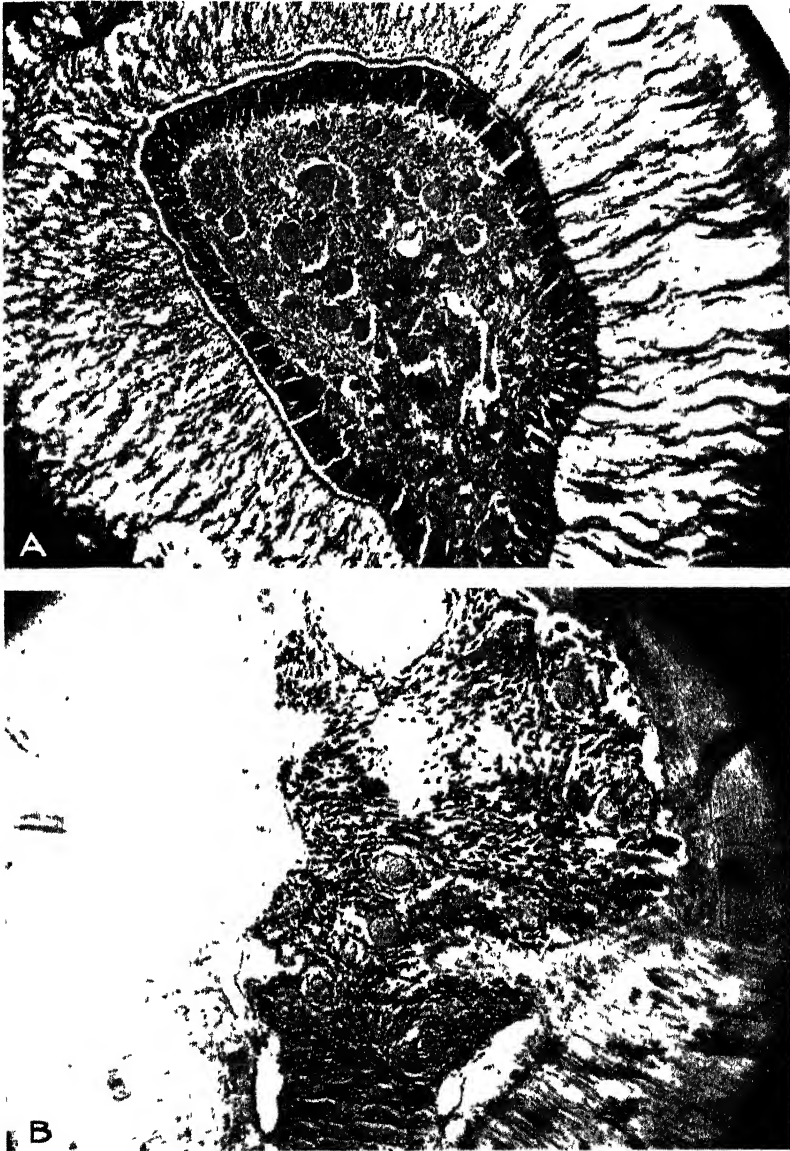


FIGURE 4.--A, Section of a normal incisor tooth taken from a guinea pig fed a diet complete in all necessary factors; B, section of a tooth from a scorbutic guinea pig that received a vitamin C deficient diet for 18 days

of evenly stained dentine inside of which is a narrow layer of uncalcified predentine, and then a row of very tall parallel columnar odontoblasts surrounding the normal pulp. An inadequate supply

of vitamin C causes the layer of dentine to become narrower, the predentine becomes calcified, and the odontoblastic layer of cells loses its soldierlike formation, while the cells themselves become shorter and gradually work their way into the pulp cavity to function as osteoblasts, the bone-forming instead of the dentine-forming cells. Consequently the scorbutic condition portrayed in the tooth structure in Figure 4, B, can be readily recognized even before the guinea pig develops outward symptoms of scurvy.

All of the teeth taken from the guinea pigs receiving commercial grape juice No. 2 showed marked pathological conditions analogous

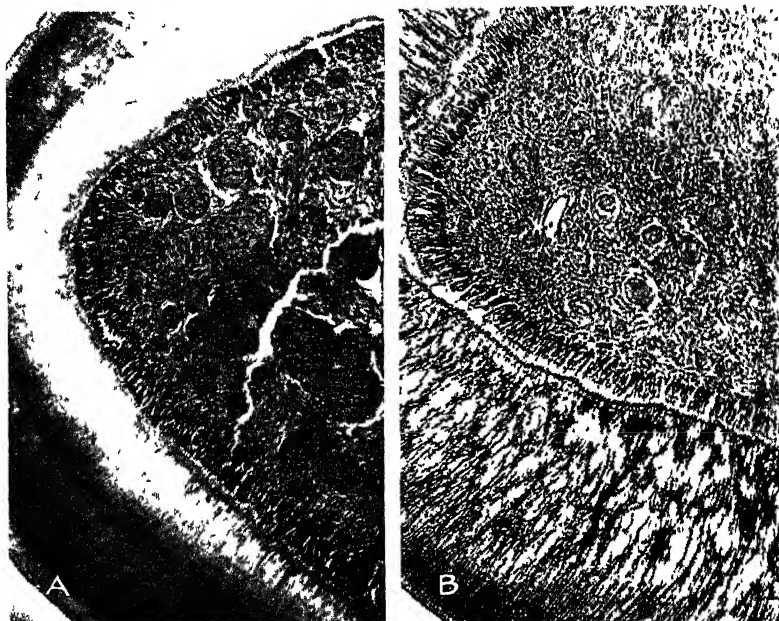


FIGURE 5—A, Section of an incisor taken from a guinea pig receiving 15 gm. of Malaga grapes as the sole source of vitamin C, B, section of a tooth from a guinea pig receiving 15 gm. of Sultanina grapes as the sole source of vitamin C

to those portrayed in Figure 4, B. Thus, it is concluded that this juice contains no appreciable amount of vitamin C.

The sections prepared from the teeth of animals fed 2-gm. and 5-gm. supplemental portions of either Malaga or Sultanina grapes showed that neither variety in these quantities prevented the occurrence of severe pathological changes in the teeth. While there was a slight protection in the teeth of guinea pigs fed 10 gm. of grapes, still this quantity furnished far too little vitamin C to give a normal tooth structure. Figure 5, A and B, representative of the teeth of those animals receiving 15 gm. daily of Malaga and Sultanina grapes, respectively, shows that even this quantity of the fruit was insufficient to afford border-line protection. Of the two varieties of fresh grapes studied, Sultanina contained the greater amount of vitamin C (fig. 5, B), 15 gm. of this fruit offered approximately the same protection as 2 c. c. of orange juice (Fig. 6).

VITAMIN G (B<sub>2</sub>)

No report of any kind has been found that gives the vitamin G content of grapes or grape juice



FIGURE 6. -Section of an incisor tooth taken from a guinea pig receiving 2 c. c. of fresh orange juice daily as the sole source of vitamin C.

A method for the determination of vitamin G was worked out in this laboratory, and is similar in some respects to that used by Sandels (11). For a period of two weeks, 28-day-old rats were given the basal diet alone in order to deplete them of any vitamin G that might

be stored in their bodies. Vitamin B was supplied in the form of an 80 per cent by weight alcoholic extract of white corn which up to the present time has been found in this laboratory to be the most satisfactory source of vitamin B free from grossly interfering amounts of vitamin G. The basal diet consisted of the following ingredients: Purified casein, 18 per cent, Osborne and Mendel salts, 4 per cent; butterfat, 8 per cent, cod-liver oil, 2 per cent, starch, 68-X per

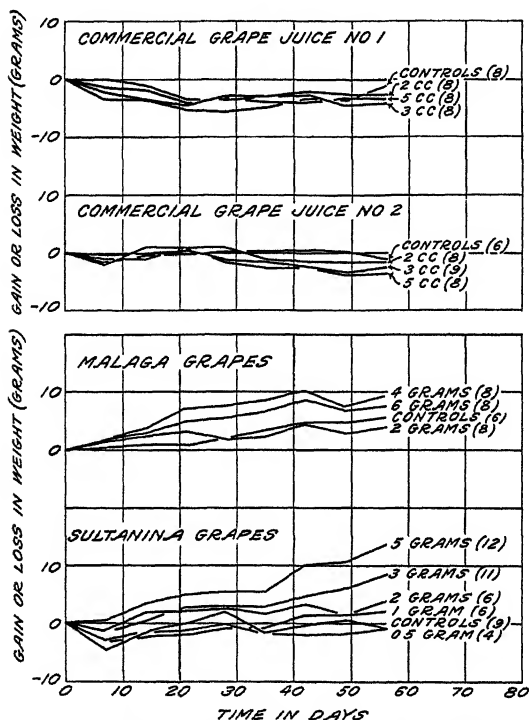


FIGURE 7.—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) and two brands of commercial grape juice (No. 1 from Flame Tokay and Zinfandel grapes, *V. vinifera*, No. 2 from Concord grapes, *V. labrusca*) as the sole source of vitamin G (B). The quantity of grapes in grams and the cubic centimeters of juice fed daily six times per week is indicated at the end of each respective curve. The numerals in parentheses indicate the number of animals subjected to each respective test.

cent,<sup>3</sup> and corn extract, X per cent. Sultanina grapes were fed to the different groups of animals in 0.5, 1, 2, 3, and 5 gm daily portions six times per week; the Malagas were given in the same manner in 2, 4, and 6 gm amounts, while each grape juice was fed in 2, 3, and 5 c c allotments. The experiment covered a period of 10 weeks, but no significant changes occurred during the ninth and tenth weeks, and therefore only 8 weeks of the test are portrayed in the curves showing the rate of growth of the animals. (Fig. 7)

The animals that received an insufficient amount of vitamin G first showed wooliness of fur and then a thinning of the hair, especially on the sides of the head. About the sixth week of the test a brownish-red discharge appeared

caked along the inner sides of the forelegs and paws, and considerable of this material appeared on the nose and whiskers of the animals. Although it strongly resembled blood, there was no sign of broken skin or bleeding on the legs directly under the deposit, and it was not possible to obtain a positive blood reaction when the benzidine test was used. Upon autopsy, the contents of the stomach and intestines appeared very similar to this discharged material. Very often lesions appeared on the side of the head out from the eyes and the corners of the mouth. The animals seemed very nervous and spent considerable time rubbing their heads. Priapism was very commonly found.

<sup>3</sup> X represents the amount of extract, evaporated on cornstarch, obtained through the extraction of 90 gm of corn.

Sultanina grapes in 5-gm daily portions induced an average gain of 13.7 gm for the entire eight weeks and therefore contain a small but significant amount of vitamin G. The animals receiving the Malagas did not make sufficient growth, as shown in Figure 7, to indicate the presence of any of this vitamin. From the same figure it may be seen that neither juice contains any vitamin G.

### SUMMARY

Fresh Sultanina (Thompson Seedless) and Malaga grapes (*Vitis vinifera*) and two brands of commercial grape juice (No. 1, a mixture of juices from Flame Tokay and Zinfandel varieties, *V. vinifera*, and No. 2, the juice from Concord grapes, *V. labrusca*) were tested for their vitamin A, B ( $B_1$ ), C, and G ( $B_2$ ) content. The results showed that:

Both varieties of grapes contained a small but measurable amount of vitamin A. There was no evidence of this vitamin in either juice.

Vitamin B (antineuritic) was present in fair amounts in both kinds of fresh grapes tested and in small quantity in the commercial juice designated as No. 2. Commercial juice No. 1 did not contain vitamin B in a measurable quantity.

Fifteen grams of fresh grapes fed daily were found to contain insufficient amounts of vitamin C to protect guinea pigs from scurvy as determined by the Hojer method. This quantity of Sultanina grapes furnished approximately the same protection as 2 c c of orange juice and contained more of the antiscorbutic vitamin than the Malaga grapes. There was no indication of vitamin C, as determined by the Sherman method, in commercial juice No. 1. Tests made by the Hojer method indicated the absence of antiscorbutic vitamin in commercial juice No. 2.

Sultanina grapes appeared to contain a minimal amount of vitamin G, while Malaga grapes and both juices were lacking in this vitamin.

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# FACTORS INFLUENCING THE CHANGES IN OXIDATION-REDUCTION POTENTIAL ON THE REDUCTION OF METHYLENE BLUE IN MILK<sup>1</sup>

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## INTRODUCTION

The methylene blue reduction test as it is used to-day is one of the most practical tests for determining the quality of milk. Although the early conceptions of biological reduction processes have been completely reorganized, the selection of the dye and the concentration employed have not been changed by a more fundamental understanding of the factors involved. The newer conception of the methylene blue reduction test is the result of studies of reducing intensities of biological systems. For the most part, these studies have been of a more fundamental and theoretical nature, with less emphasis upon their practical application to the reductase test.

In this paper an attempt is made to correlate the factors influencing the changes in oxidation-reduction potential with the reduction of methylene blue in milk.

## LITERATURE REVIEW

Fred (15)<sup>2</sup> presented an excellent historical review of the early literature pertaining to dye reduction by microorganisms. As a result of his own researches, Fred firmly established the dependence of reduction time of methylene blue in milk on the quantitative and qualitative aspects of the original bacterial flora. The probability that the reduction of methylene blue might be due to some constituent of the milk was suggested by Barthel (1) and Hastings (18).

The first evidence that the reducing intensity of bacterial cultures might be measurable in terms of electrode potential was presented by Gillespie (16). In measuring the reduction potentials of bacterial suspensions and of water-logged soils, he observed a trend toward more negative reducing intensities.

Clark (5) measured the equilibrium potentials of the systems methylene blue--methylene white and indigo-indigo white. As a result of these studies, he established quantitative values for the different reducing intensities indicated by these systems.

Following a study of the significance of anaerobiosis, Hall (17) states that adsorption plays an important rôle in the decolorization of dyes by porous substances such as animal and plant tissues.

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 82.

Hastings, Davenport, and Wright (19) conclude that the reduction of methylene blue is very intimately connected with the vital processes of the cell rather than with any extracellular by-products.

By microinjection of several reversible oxidation-reduction indicators into *Amoeba proteus* and *A. dubia*, Needham and Needham (22) and Cohen, Chambers, and Reznikoff (8) found that these protozoa maintain a fairly constant reduction potential.

In 1920 Clark (5) presented a comprehensive basis for interpreting, in terms of electrode potential, the results given by biological reduction of reversible oxidation-reduction systems. Clark and his associates (6) made a quantitative study of the potential of a large number of the oxidation-reduction indicators, including methylene blue, and determined the relative position of these indicators on the potential scale. They presented the time-potential curves of samples of inoculated, bottled, and fresh milk.

By measuring the potentials of cells, extracts, and cultures, Cannan, Cohen, and Clark (3) showed a general correlation between the reduction potential of a cell suspension, the cellular reduction of a dye, and the reduction potential of the same dye as determined in pure solution. They showed also that different species of bacteria attain different levels of reducing intensity and follow different courses.

Coulter (9) observed the parallelism between the reducing intensities induced by bacterial respiration and those attained by the removal of oxygen from sterile bouillon. He concluded that the development of the characteristic negative limits of intensity in bacterial cultures can not be attributed entirely to reductive processes directly dependent upon the action of living cells.

Cohen (7, p. 16-17) states

Bacterial cultures in broth and synthetic media develop progressively increasing reducing intensities which have been followed electrometrically. Oxidation-reduction indicators, within the limits imposed by chemical reactivity and narrow useful range confirm the time-potential curves. The levels of reduction potentials attained by cultures of different bacteria are more or less different and characteristic.

Sterile broth when protected from the atmosphere by a vaseline seal is capable of reducing a number of dyes, including methylene blue, as demonstrated by Dubos (10).

Thornton and Hastings (24) observed a very close similarity between the potential-time curves of milk with and without methylene blue. Although the potentials of the zone of visible reduction of methylene blue in milk were found to be variable, they were always more positive than the theoretical zone in pure solutions of this dye at the same pH value. These authors were able to decolorize the dye in milk by deaeration and to restore the blue color by aeration. They state (25) that their work tends to confirm Barthel's (2) theory of methylene blue reduction in milk.

It was shown by Fildes (11) that the period required for the germination of spores of *Bacillus tetani* depends mainly on the time required for the medium to reach a suitable reducing intensity. The same writer (12) reported that the subcutaneous tissues of a living guinea pig maintain an  $E_h$  on the positive side of reduced methylene blue, and that the  $E_h$  becomes more negative at the death of the animal.

Lepper and Martin (21) reported that cooked-meat media when exposed to air was reduced by cultures of two aerobes and five ana-

erobes Hewitt (20) measured the potentials of three cultures in several kinds of medium, and found that *Corynebacterium diphtheriae* and *Staphylococcus aureus* were usually able to attain more negative reducing intensities than a hemolytic streptococcus.

### METHODS

Burnished platinum foil electrodes were chosen after an extensive comparison of results obtained in parallel tests with gold foil, gold wire, platinum wire, and gold-plated platinum electrodes. Electrodes 1 cm. square were submerged to a depth of about 2 inches in approximately 50 c. c. samples. By means of suitable switches leads from six electrodes were connected to a Leeds and Northrup type K potentiometer. A saturated KCl calomel half cell was used as the reference electrode. Connections were made from the reference electrode to the

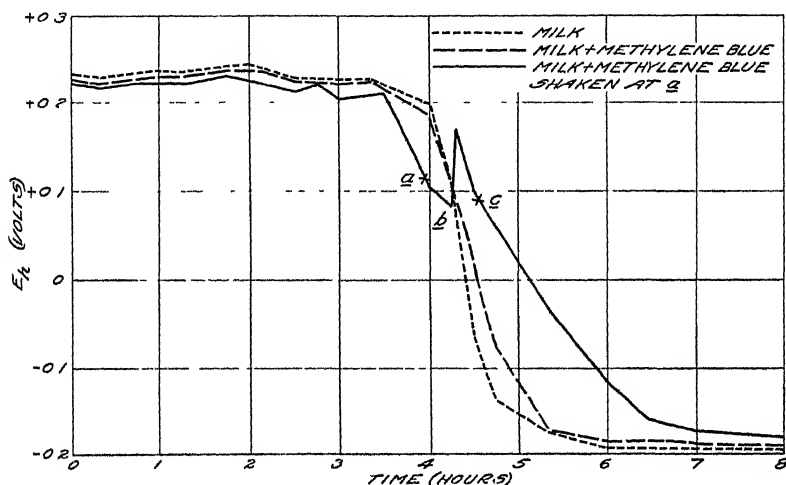


FIGURE 1.—Potential-time curves of a sample of market milk with and without the addition of methylene blue

samples under measurement by means of a saturated KCl liquid junction and saturated KCl-agar bridges. Samples were incubated in a water bath maintained at  $37^{\circ} + 1^{\circ}$  C. Potential readings taken at suitable intervals were reduced to the hydrogen standard ( $E_h$ ) and plotted as the ordinate against time as the abscissa.

### EXPERIMENTAL DATA

#### EFFECT OF THE PRESENCE OF METHYLENE BLUE IN MILK ON THE FORM OF THE POTENTIAL-TIME CURVES

A sample of market milk was divided into three parts, to two of which was added the standard amount of methylene blue ordinarily employed in the reductase test (1:200,000). The oxidation-reduction potentials of these three identical samples were followed through the entire course of the reduction process. The marked similarity of the potential-time curves (fig. 1) of the samples with and without methylene blue suggests that this concentration of dye has no marked effect upon the trend of the potential drift.

The effect of incorporating oxygen by shaking is illustrated in Figure 1 by the solid line. The blue color had completely disappeared at point *a*, and at point *b* the sample was shaken vigorously for 30 seconds. The return of the potential to approximately the original positive values was accompanied by a return of the blue color. The color had again disappeared at point *c*. The second drop in potential in this sample was not so rapid as that occurring in the other two samples, probably because of the deterring effect of the incorporated oxygen on potential drift. Attention is called to the marked similarity of the curves in Figure 1 with those published by Thornton and Hastings (24) illustrating a similar experiment. The form of the potential-time curves, the zones of decolorization, the effect of incorporated oxygen, and the negative limits attained are almost identical with the results obtained by Thornton and Hastings.

In further studies, air was bubbled into a sample of milk plus methylene blue after the potential had reached the negative  $E_h$  limit of  $-0.2$  volt. The potential returned almost to the positive extreme, but the blue color did not return. The potential was observed 30 minutes after the positive extreme had been reached and was found to be falling rapidly to the negative side.

#### EFFECT OF THE BACTERIAL FLORA IN MILK ON THE FORM OF THE POTENTIAL-TIME CURVES

Clark and his associates (6) and Frazier and Whittier (13, 14) reported that various species of bacteria run characteristic courses and attain different levels of reducing intensity, thus giving rise to various though characteristic forms of potential-time curves. The results of these investigators suggest a plausible explanation for some of the difficulties commonly encountered in the practical application of the methylene blue reduction test. Frequently, the time elapsing between the first evidence of diminution of color and complete decolorization of the dye is so prolonged as to render the end point very indistinct. It is not uncommon to find samples of milk in which 30 to 60 minutes elapse between the beginning and the end of visible reduction of methylene blue. Apparently this is due to the type or types of organisms which dominate the flora of the milk.

In order to determine whether the variations reported in the literature on pure culture studies could be reproduced with the mixed flora of market milk, the oxidation-reduction potentials of samples incubated at various temperatures were noted. It was commonly observed that most fresh milk gave a potential-time curve which fell rapidly through the zone of reduction of methylene blue in less than five minutes, whereas for the same milk after 48 hours at  $3^{\circ}$  to  $5^{\circ}$  C the time elapsing between the beginning and the end of visible reduction frequently exceeded 30 minutes. Although the trend of the curves obtained from samples incubated at higher temperatures were quite variable, the results emphasize the significance of the dominating organisms in the flora as a factor which may be responsible for the slow reduction of the dye in some samples of milk. Plotting the potential drift of a large number of samples of milk has shown considerable variation in the form of these curves. The curve for a sample of fresh milk is characterized by a rapid fall from the positive to the negative extremes. If a sample of milk

giving rise to this form of curve contains the standard amount of methylene blue, the interval between the beginning and the end of visible reduction will be short, usually less than five minutes. It was commonly observed that the bacterial flora of milk held 48 hours at 3° to 5° C. gave a potential · time curve which fell slowly to the negative extreme. This was accompanied by a slow decolorization of the methylene blue, frequently observed to extend over a period of 30 minutes. It is evident that a rapidly falling potential will pass through the zone of visible reduction in less time than one that falls slowly, thus explaining the variations in time required for decolorization of the dye in different samples of milk.

#### EFFECT OF FAT ON THE ZONE OF REDUCTION OF METHYLENE BLUE

It was noted that when the standard amount of methylene blue was added to skim milk the dye decolorized between the  $E_h$  values of zero and +0.05 volt. The potentials of this zone are approximately 0.1 volt more negative than the zone of decolorization of the same amount of methylene blue when added to whole milk. It was also observed that the same amount of methylene blue, when added to cream, decolorized between the  $E_h$  limits of +0.3 and +0.2 volt. The potential of this zone is approximately 0.1 volt more positive than that observed for whole milk.

To determine more definitely the potential of the zone of reduction of methylene blue in milk of various percentages of fat, sterile 40 per cent cream and sterile skim milk were mixed in suitable proportions to obtain six solutions containing 40, 30, 20, 10, 5, and 0 per cent of fat. The solutions were inoculated equally with a 24-hour culture of *Streptococcus lactis*, and the standard amount of methylene blue was added to each. The oxidation-reduction potentials were followed, and the potential · time curves of the six solutions are presented in Figure 2. The potentials of the zone of reduction of the methylene blue are indicated by triangles at the right of the respective graphs.

The potentials of the zone of reduction of methylene blue in skim milk are more negative than those observed in the case of cream. Methylene blue was reduced in skim milk between the  $E_h$  values of +0.092 and +0.050, and in cream between the values of +0.275 and +0.245. The zones of reduction in the other samples, without exception, became more positive as the percentage of fat was increased. The potentials of the zone of reduction of methylene blue in skim milk approximate more closely the theoretical zone for this dye in aqueous solution as reported by Clark and his associates (6).

The potentials of more than 25 samples of skim milk have been measured, and in no case has the methylene blue been observed to be reduced at a potential more positive than +0.1 volt. The zone of reduction of methylene blue in 50 samples of 40 per cent cream was never observed to be more negative than +0.225 nor more positive than +0.3 volt. It may be noted that the form of the potential · time curves is not affected by varying the percentage of fat.

Other factors being equal, it would require a somewhat longer time to reduce methylene blue in skim milk than in 40 per cent cream with the same original bacterial content. In the case of skim milk the oxidation-reduction potential must be carried to the negative

limits of approximately  $-0.05$  volt, whereas in the case of 40 per cent cream visible reduction is usually complete at an  $E_h$  of  $+0.25$  volt.

The exact manner in which fat alters the zone of reduction is not known. Studies of oxidation-reduction phenomena have been limited largely to simple equilibria in aqueous solutions. Many of the fundamental aspects of the simplest systems are yet to be understood. The present status of our knowledge of these simple equilibria certainly does not encourage speculations with respect to complex systems of unknown composition, as is the case with biological fluids. The more positive zone of reduction of methylene blue in cream than in skim milk may involve some unknown factors in oxidation-reduction equilibria. The work of Hall (17) suggests the possibility that adsorption of the dye may play a rôle in this connection.

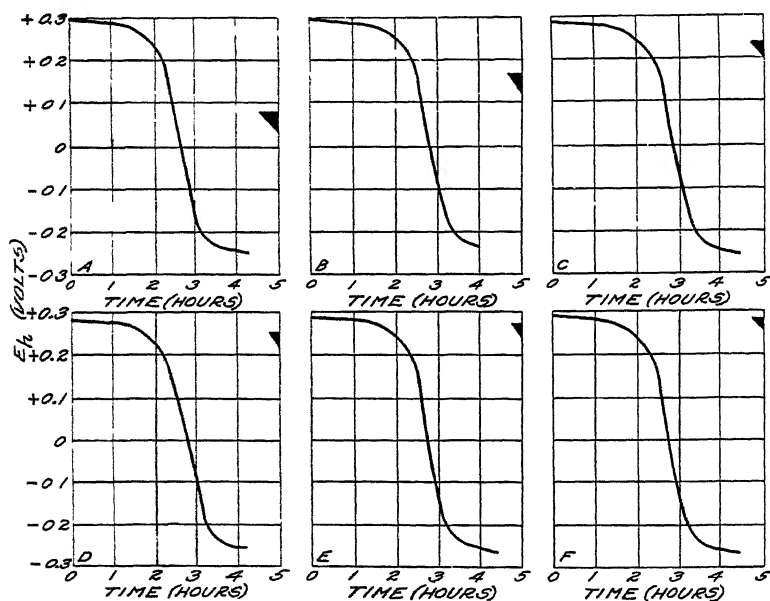


FIGURE 2.—Relation between the percentage of fat in milk and the zone of reduction of methylene blue. A, Skim milk, B, 5 per cent fat, C, 10 per cent fat, D, 20 per cent fat, E, 30 per cent fat, F, 40 per cent fat. Potentials of the methylene blue zone of reduction are indicated by black triangles.

Cursory experiments indicate that approximately four times as much methylene blue must be added to 40 per cent cream to give the first perceptible tinge of blue as is required for skim milk. Conversely, in the decolorization of the dye, the point at which color is no longer discernible will be reached sooner in cream than in skim milk. The addition of the standard amount of methylene blue imparts a distinct blue color to skim milk, but only a very faint blue tinge to 40 per cent cream. The loss of only a slight amount of the blue dye by reduction in cream results in a disappearance of color. Obviously, this point of visible reduction will be reached very soon after the potential begins its swing toward more negative values. In the case of skim milk the blue color persists until more negative potentials have left only a relatively small percentage of the dye in the oxidized or blue form. Although this is not offered as a complete

explanation of the more positive zone of reduction of methylene blue in cream, the quantity of dye necessary for colorization or decolorization must be taken into consideration

If the detection of color is dependent on a requisite minimum number of molecules of the oxidized form of the dye, one would expect the addition of larger amounts of methylene blue to lower the zone for cream to the approximate  $E_h$  values observed for skim milk. The results of experiments in which various amounts of dye were added are presented in Figure 3

#### EFFECT OF THE CONCENTRATION OF DYE ON OXIDATION-REDUCTION POTENTIALS

##### ZONE OF VISIBLE REDUCTION

The zone of reduction of cream and skim milk may be moved up and down the potential : time curve at will by the addition of various quantities of dye. In Figure 3, curves A, B, C, and D are representative of many experiments made to determine this point

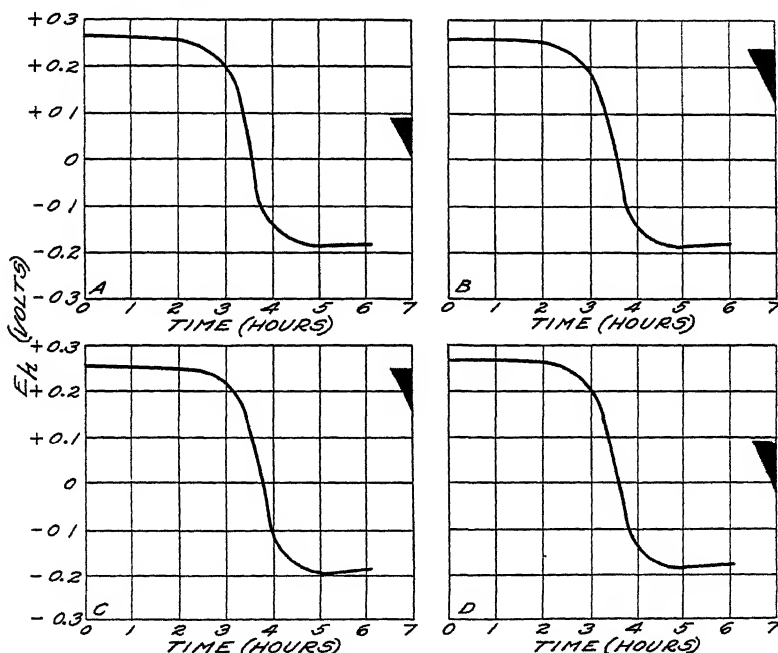


FIGURE 3 —Relation between the concentration of methylene blue used and the zone of reduction in skim milk and in 40 per cent cream. A, 1 200,000 of methylene blue in skim milk, B, 1 200,000 of methylene blue in cream, C, 1 16,000,000 of methylene blue in skim milk, D, 1 10,000 of methylene blue in cream. Potentials of the methylene blue zone of reduction are indicated by black triangles

Curves A and B show the zone of reduction of methylene blue in skim milk and cream, respectively, when the standard amount of dye (1:200,000) is added

By adding only 1 part of dye to 16,000,000 parts of skim milk the zone was changed to approximate that of cream (B). Similarly, D shows that the addition of 1 part of dye to 10,000 parts of cream caused the zone of reduction to approximate the  $E_h$  limits which apply to skim milk when the standard amount of dye is added.

## REDUCTION TIME

In recent years there has been some controversy in regard to the effect of various concentrations of dye on the reduction time of milk. Three portions of a sample of milk containing the following concentrations of methylene blue were studied potentiometrically, (A) 1 400,000, (B) 1 200,000, and (C) 1 100,000. The potential time curves of these three samples and the zones of reduction are shown in Figure 4. The three curves are so similar in form that they would almost superimpose if plotted upon the same ordinates. The zones of potential within which the methylene blue is reduced are shown by means of triangles. The position of these zones varies with the concentration of dye. In curve B, representing the sample containing the normal concentration of dye, decolorization took place in the zone between +0.225 and +0.165 volt, and was complete after 75 minutes of incubation. Curve A represents the sample containing

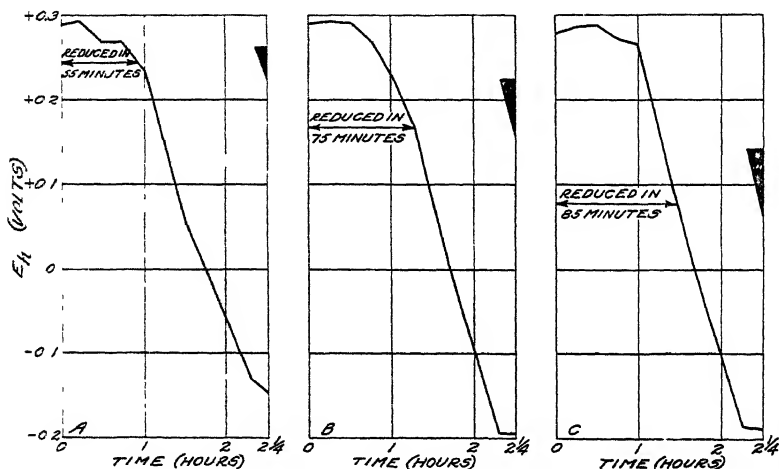


FIGURE 4—Effect of varying the concentration of methylene blue in milk upon the reduction time. A, 1 400,000 of methylene blue, B, 1 200,000 of methylene blue, C, 1 100,000 of methylene blue. Potential of the methylene blue zone of reduction are indicated by black triangles.

one-half the normal amount of dye (1 400,000). The zone of decolorization was 0.075 volt more positive than when the usual concentration of dye was used (curve B). Coincident with the more positive zone, the reduction time was shortened from 75 to 55 minutes. Curve C shows the effect of adding twice the usual concentration of dye (1 100,000). The zone of decolorization of methylene blue in this sample was 0.09 volt more negative than in the sample containing the usual concentration of dye. The time required for the reduction of the dye was increased to 85 minutes as compared with 75 minutes for sample B.

The significant aspect of these three potential time curves is the potential of the zone of decolorization of the various concentrations of methylene blue. If it be assumed that the color disappears when less than an arbitrary minimum number of molecules of the blue dye are present, the explanation of the effect of the various amounts of dye on reduction time becomes simple. If more than the normal

amounts of dye are present, more negative potentials must be reached before decolorization is effected, and hence a longer time is required. Similarly, less time would be required to attain the slightly negative potential necessary to effect decolorization of sample A (Fig 4) In other words, the more dye there is present the longer is the time required to reach a potential sufficiently negative to diminish the quantity of the dye in the oxidized form below the amount requisite for coloring

In the concentrations employed (fig 4) the dye does not affect the course of potential change, as is evidenced by the similarity of the three curves

#### FORM OF POTENTIAL TIME CURVE

The studies in the preceding experiments on the relationship of the concentration of dye to other factors were confined, for the most part, to higher dilutions of methylene blue In the following experi-

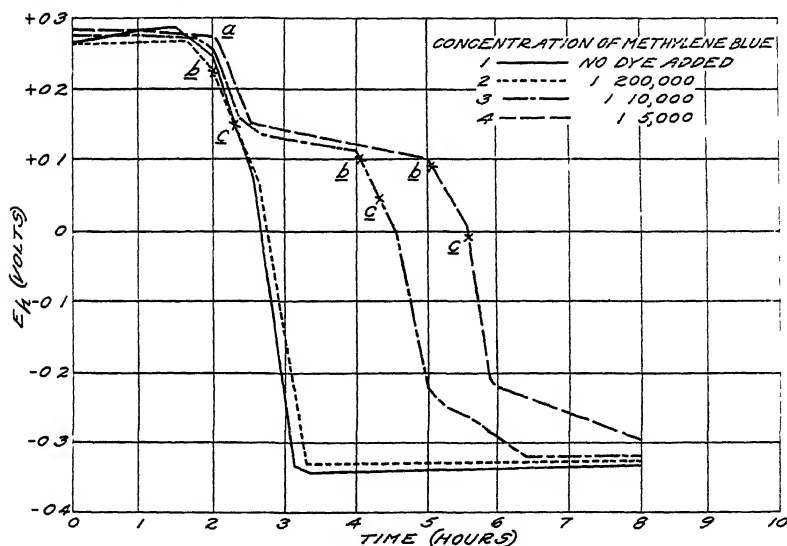


FIGURE 5—Effect of varying the concentration of methylene blue in 20 per cent cream upon the reduction time of methylene blue

ment the effect of more concentrated solutions of dye has been studied The curves in Figure 5 show the potential drift of four portions of a sample of 20 per cent cream containing the following concentrations of methylene blue: (1) No methylene blue, (2) 1:200,000, (3) 1:10,000, and (4) 1:5,000 The zones of potential within which the methylene blue reduced are indicated by the letters *b* (began) and *c* (completed) The curves of samples 1 and 2 are similar to those in Figure 4 and show that the addition of the normal amount of methylene blue does not alter the form of the potential time curve The zone of reduction of the dye in sample 2 was between the  $E_h$  values of +0.24 and +0.20 volt The potentials of this zone are similar to those previously observed (fig 4) for 20 per cent cream containing the standard amount of dye.

The potential curves of samples 3 and 4 illustrate clearly the effect of adding excessive amounts of methylene blue. There are several significant aspects of these curves which not only show the effect of the addition of excessive amounts of dye, but possibly throw some light on the mechanism of dye reduction in milk.

In the first place, all four samples began their swing toward the negative potentials simultaneously. Whether the initial fall in potential is directly or indirectly the result of bacterial activity, these results indicate quite clearly that at least the highest concentration of dye employed (1:5,000) did not exert any antiseptic action.

The plateaus observed in curves 3 and 4, especially when contrasted with the total absence of a plateau in curve 2, emphasize the fact that the poisoning effect of the dye is directly dependent upon the amount of dye added. Clark (6, p 10) defines poisoning as follows: "A solution may be said to be poisoned when it tends to resist a change in  $E_h$  on addition of an oxidizing or reducing agent."

As the potentials of the four samples of milk began their initial swing (point *a*) toward negative values, they followed the same general course until well within the zone of reduction of methylene blue. The potential drift was not impaired in sample 1 without dye, or in sample 2 in which the standard concentration of 1:200,000 was employed. In samples 3 and 4, however, the large amounts of methylene blue added exerted a poisoning effect which was directly related to the quantity of dye added.

Since the usual concentration of dye employed in the reductase test does not materially affect the oxidation-reduction system, the methylene blue simply serves as a visible indicator that this swing toward more negative potentials has taken place. As the visible reduction occurs shortly after the swing toward more negative potentials begins, the loss of color of the dye indicates that the bacterial activity has overcome the poisoning action of the oxidation-reduction system or systems of the milk. (Point *a* has been reached.)

The time required for visible reduction became progressively greater as the concentration of dye was increased. For the samples reported in Figure 5 the dye concentrations and the reduction times were as follows:

- (2) 1:200,000—128 minutes.
- (3) 1:10,000—250 minutes
- (4) 1:5,000—335 minutes

The reduction of dye in samples 3 and 4 was not completed until after the second drop in the potential had started. The data in Figure 5 further substantiate the observations made in connection with Figure 3, viz, that the amount of dye employed affects the zone of reduction.

#### EFFECT OF THE CONCENTRATION OF SUGAR ON OXIDATION-REDUCTION POTENTIALS IN CREAM

There is a demand at the present time for a practical test to determine the sanitary quality of dairy products such as ice cream and ice-cream mixes. Early in the course of these experiments, attempts to follow the course of the potential drift of ice cream showed that the curve tended to pass slowly toward negative values. The visible reduction of the dye was correspondingly delayed over an

extended period Attempts to determine the cause of the peculiar nature of the curve led to a series of experiments to demonstrate the effect of sugar on the potential drift Figure 6 shows the results of a typical experiment

Sterile cream, skim milk, and a cane-sugar solution were combined in suitable proportions to give various concentrations of sugar (0, 10, 20, and 25 per cent) and a constant fat content of 20 per cent Each sample was inoculated with a 24-hour culture of *Sireptococcus lactis* and the standard amount of dye was added The form of the potential time curves was markedly altered by increasing the percentage of sugar Increasing the amount of sugar delayed the potential trend to more negative values, which in turn lengthened the reduction time Although equal amounts of inoculum were added to each sample, obviously the number of bacteria added could not be accurately

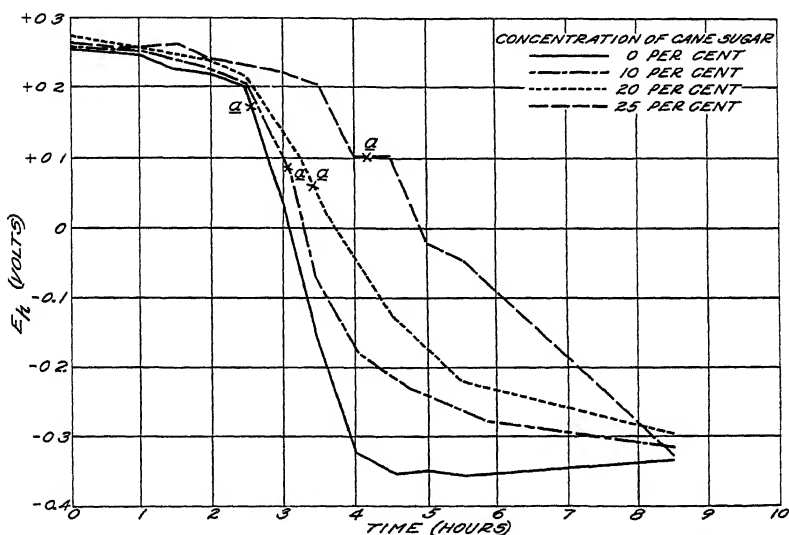


FIGURE 6—Effect of varying the concentration of cane sugar in cream-skim milk ice-cream mixes upon the reduction time of methylene blue Reduction occurred at point *a* on each curve The fat content of the mix was 20 per cent

controlled Nevertheless, the time required for reduction was increased directly with larger amounts of sugar The reduction times for the samples in the order of increasing amounts of sugar were 155, 185, 205, and 248 minutes, respectively The differences in the form of the potential time curves may have been due to a change in the metabolic activities of the cells, although evidence to support this suggestion is not available It has been shown by Hewitt (20) that changes in the medium affect the reduction intensities attained by bacterial cultures It is of interest to note the extreme negative levels ( $-0.3$  and  $-0.35$  volt) attained by these cultures Clark and his associates (6), Rogers and Whittier (23), and also Frazier and Whittier (13) have shown that cultures of *S. lactis* in milk usually reach a negative limit of approximately  $-0.2$  volt.

## SUMMARY

The potential-time curves of milk with and without methylene blue remained in close agreement during the entire reduction process. The blue color and initial potentials of reduced samples could be restored by vigorous shaking or aspirating with air. Either of the above treatments also restored the initial potentials of samples without dye.

The bacterial flora of a sample of market milk influences the form of the potential-time curve.

The position of the zone of visible reduction was caused to vary by altering either the fat content of the sample or the concentration of the dye added. The zone of reduction became more positive with an increase in the percentage of fat and more negative with an increase in the concentration of dye.

The time required for visible reduction increased as the zone of reduction became more negative.

When excessive amounts of dye (1:10,000) were added, the potential of the solution did not pass smoothly to more negative limits, but was deterred as it approached the zone of reduction characteristic of this indicator.

The addition of cane sugar to cream not only delayed the potential drift and reduction time of the dye, but affected the form of the potential-time curve as well.

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# EFFECT OF LIGHT ON THE REDUCTION OF METHYLENE BLUE IN MILK<sup>1</sup>

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## INTRODUCTION

In a previous paper (2)<sup>2</sup> it has been shown that methylene blue in milk changes to methylene white when a certain specific zone of potential is reached. In the concentrations employed in the reductase test the dye apparently exerts no effect on the potential drift, and therefore serves merely as a visible indicator that a definite reducing intensity has been attained.

For a number of years it has been known that a medium such as milk or broth containing methylene blue loses its color when exposed to sunlight. It has not been determined, however, whether sunlight merely bleaches the dye directly or causes a change in the reducing intensity of the substrate comparable to that induced by bacteria. The purpose of studying the effect of light on the reduction of methylene blue was to establish more firmly the correlation between reducing intensities and dye reduction, and thereby afford a more intelligent approach to the fundamental mechanism of such reactions.

## LITERATURE REVIEW

Gebhard (5), and Lasareff (8) have shown that the bleaching effect of light on methylene blue is most intense in the absence of oxygen provided the available light consisted of waves shorter than  $620\ \mu$ , the color returned in the dark in the presence of oxygen.

In connection with his studies on anaerobiosis, Hall (7) observed that light induced the decolorization of methylene blue when added to broth cultures.

Whitehead (11) summarizes his work on methylene blue reduction in sunlight as follows.

1 Methylene blue added to fresh milk of good quality is reduced in a short time in the presence of sunlight at  $37^{\circ}$  \* \* \*

2 The reduction in sunlight is not due to an enzyme \* ~ \*

3 Milk from which the fat has been removed by centrifugal separation no longer gives the reaction, but the activity of the milk can be restored by an addition of sodium oleate. Sodium palmitate has not a similar action.

4 It is suggested that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidized and methylene blue is reduced.

In a discussion of the theoretical aspects of oxidation and reduction of colored compounds Michaelis (9, p. 69) suggests that they may all have loose electrons which render them peculiarly adapted to oxidation-reduction systems in the presence of suitable electron acceptors. The decolorized dye likewise may acquire electrons from still more powerful reductants. These properties not only facilitate their rôle in reversible oxidation-reduction systems, but may explain the fre-

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 95.

quently observed catalytic action of such compounds in oxidations. Michaelis further suggests that although these theories are not well established they may account for the effect of radiant energy on the instability of electrons

### METHODS

In order to correlate the reduction of methylene blue in milk by light and the changes in reducing intensity, samples of milk were subdivided so as to give replicates with and without dye, exposed, and unexposed to light. The potential drifts in the various samples were followed by means of the apparatus described in a previous paper (2). In order to afford a proper exposure to the sun's rays test tubes containing the samples were submerged to a depth of about 1 inch in a water bath maintained at a suitable temperature. Because of the prevailing low outdoor temperature during most of the experiments it was not feasible to place the water bath in an open window, and

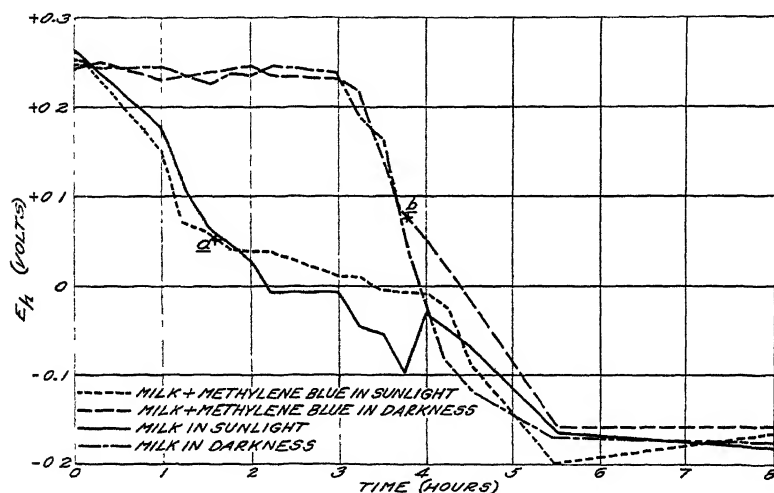


FIGURE 1—Effect of sunlight on the potential time curves and reduction time of market milk with and without the addition of methylene blue, complete reduction took place at points *a* and *b*

therefore the sunlight filtered through a window pane in addition to the walls of the test tube. It may be assumed that the effective light did not contain rays shorter than approximately  $340 \mu$ , the value usually given for the transmissibility of ordinary glass.

### EXPERIMENTAL DATA

EFFECT OF SUNLIGHT ON THE OXIDATION-REDUCTION POTENTIAL OF MARKET MILK, CREAM, SKIM MILK, AND SKIM MILK PLUS SODIUM OLEATE AND SODIUM STEARATE

It was consistently observed that in the tubes exposed to sunlight the dye was reduced within 15 to 90 minutes, the length of time depending upon the intensity of the sunlight. It was also generally observed that increasing the percentage of fat shortened the time required for reduction of the dye. Similar results were obtained by adding increasing amounts of sodium oleate to skim milk. In order to obtain a more complete history of the changes occurring in milk,

cream, and skim milk exposed to sunlight, the oxidation-reduction potentials of a number of samples were measured at suitable intervals

#### MARKET MILK

Ten cubic centimeter samples of market milk were placed in each of four sterile test tubes and the standard amount of methylene blue (1:200,000) was added to two of them. One tube of milk containing methylene blue and one without dye were placed in the sunlight, the two remaining tubes were covered with a sleeve of heavy black paper.

Figure 1 shows that the potentials of the samples exposed to sunlight became more negative immediately after exposure. This negative drift continued until an  $E_h$  value of approximately zero was reached. The milk containing the methylene blue was completely reduced at an  $E_h$  value of +0.065 volt (point *a*). After the initial rapid fall the potentials remained at an  $E_h$  value of approximately zero for three hours, or until four hours after the beginning of incubation. At this time the potentials of the four tubes came into close agreement. The potentials of the two tubes not exposed to sunlight had retained their initial  $E_h$  values for three hours, at which time they began to fall rapidly to the negative side. The potential drift of the milk in the dark may well be attributed to bacterial activity.

Gillespie (6) first suggested that bacterial cultures may induce a negative drift of electrode potential. Clark and his associates (1), Thornton and Hastings (10), and Frazier and Whittier (3, 4) have studied the effect of bacterial cultures upon the electrode potential of milk.

Attention is called to the fact that the four curves (fig. 1) tend to converge at an  $E_h$  value of approximately zero. This is considerably more positive than the ultimate negative limit of the potential drift (-0.2 volt). A comparison of these curves shows quite clearly that the light was unable to lower the potential below the  $E_h$  value of approximately zero. After these curves converged with those of the two tubes kept in the dark, all remained in close agreement throughout the remainder of the reduction process. The visible reduction of the sample in the light (*a*) preceded that of the one in the dark (*b*) by 2.5 hours.

The samples in the dark and in the light showed complete visible reduction (points *a* and *b*) at approximately the same  $E_h$  value. This suggests that the reduction of the standard quantity of methylene blue (1:200,000) in milk takes place within a definite potential zone, and that the change of color occurs whenever this potential is reached, whether the potential drift be induced by physical or biochemical processes. Parallel determinations of the hydrogen-ion concentration of samples exposed and protected from the light indicate that the variations in potential drift could not be accounted for on a basis of changes in pH.

#### CREAM

The effect of sunlight on the oxidation-reduction potentials of four portions of a sample of 40 per cent cream with and without dye were studied in the same manner as in the preceding experiment. The potential:time curves are shown in Figure 2. As in the case of milk, sunlight induced a negative potential drift immediately after exposure, whereas the  $E_h$  value of the samples in the dark remained con-

stant for several hours. In this experiment, however, the presence of higher concentrations of fat apparently affected the ability of the light to induce more negative  $E_h$  values. The initial potential drift in the two samples (with and without dye) exposed to sunlight is extended over a considerable period, in contrast to the rather sudden fall observed for whole milk (Fig 1). Also, a comparison of curves for the samples exposed to sunlight shows that when methylene blue is present sunlight is able to induce more negative potential values than when no dye is added to the cream.

As in the preceding experiment, the potentials of the zones of visible reduction (*a* and *b*) are essentially the same for samples in the light and in the dark. The presence of fat tends to elevate the zone of reduction, whether induced by bacterial action or sunlight. The fact that increasing percentages of fat shorten the reduction

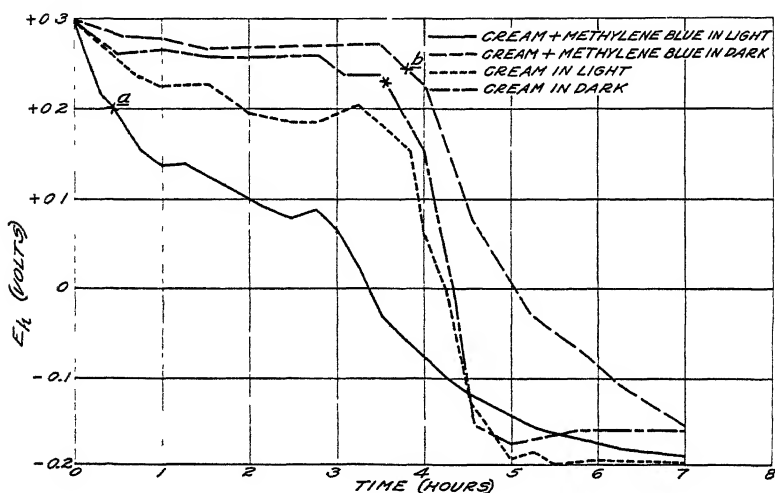


FIGURE 2—Effect of sunlight on the potential time curves and reduction time of cream (40 per cent fat) with and without the addition of methylene blue, complete reduction took place at points *a* and *b*

time of exposed samples is apparently due to the tendency of fat to elevate the zone of reduction.

#### SKIM MILK

The oxidation-reduction potentials of skim milk with and without dye exposed to the light and in the dark were followed in exactly the same manner as for cream and whole milk. The potential time curves of the samples of skim milk are shown in Figure 3. The curves are similar in a general way to those presented in Figures 1 and 2 for whole milk and cream. However, sunlight causes a greater and more rapid fall of potential in the skim milk than in either cream or whole milk. Nevertheless, the lower zone of reduction in the skim milk lengthens the time required to attain the reducing intensity necessary for complete loss of color. As in the case of cream, the addition of dye to the skim milk enabled the sunlight to induce a more negative potential drift than in the same skim milk without dye. In harmony with previous observations, as shown in Figures 1 and 2, the  $E_h$

values at the time of complete visible reduction (*a* and *b*) were essentially the same, whether induced by bacteria or sunlight. The potentials of the four samples came into close agreement after 3.5 hours and remained together during the remainder of the reduction process.

#### SKIM MILK PLUS SODIUM OLEATE AND SODIUM STEARATE

In the preliminary studies on the reduction of methylene blue by sunlight, it was observed that skim milk containing sodium oleate and methylene blue was readily reduced. In order to determine the effect of such substances on the potential of the zone of reduction of methylene blue, a sample of skim milk was divided into three parts and treated as follows: (1) 1 per cent sodium oleate, (2) 1 per cent sodium stearate, and (3) not treated. The standard amount of methylene blue was added to each of the three samples. The potential, time curves and points of complete visible reduction of the three

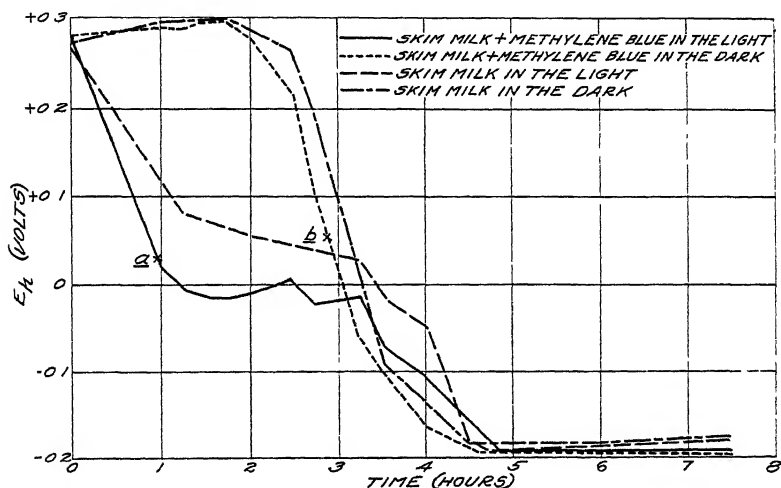


FIGURE 3—Effect of sunlight on the potential time curves and reduction time of skim milk with and without the addition of methylene blue, complete reduction took place at points *a* and *b*

samples are shown in Figure 4. The potentials of the samples containing the fatty acid salts drifted toward the negative side more rapidly than they had in the case of skim milk. The potentials of these two samples dropped rapidly to  $E_h = -0.025$  volt, after which they remained fairly constant. After the samples had incubated for four hours no more sunlight was available and the potentials returned to the positive side. The potentials remained in close agreement throughout the remainder of the reduction process. After seven hours the potential of each sample quickly drifted to the negative limit of approximately  $-0.2$  volt, induced, no doubt, by bacterial activity.

The letters *a*, *b*, and *c* on Figure 4 represent the points at which the methylene blue was completely decolorized. The zone of reduction of methylene blue is evidently not affected by the presence of either of the fatty acid salts employed in this experiment. Not only does the uniformity of the  $E_h$  values at the time of reduction of the

dye in the three solutions (*a*, *b*, and *c*) emphasize this fact, but the values conform to those previously observed for the zone of reduction of this dye in skim milk (approximately  $-0.025$  volt). Any deterring influence which butterfat may have exerted on changes in potential in the preceding experiments, apparently is not induced by 1 per cent of sodium oleate or sodium stearate.

It was observed in a preceding experiment (fig. 2) that the presence of butterfat shortens the reduction time of exposed samples by elevating the zone of reduction. The tendency of sodium oleate and sodium stearate to shorten the reduction time is apparently due to their ability to accentuate the potential drift. The contrast between the action of butterfat and these readily oxidizable substances may throw some light on the mechanism of dye reduction.

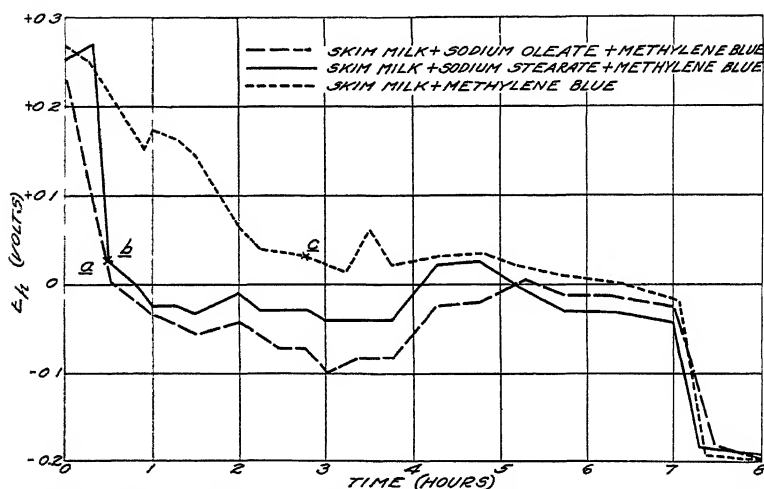


FIGURE 4—Effect of sunlight on the potential time curves of skim milk, skim milk plus sodium oleate, and skim milk plus sodium stearate, with the addition of methylene blue, complete reduction took place at points *a*, *b*, and *c*.

#### EFFECT OF CONCENTRATION OF DYE ON THE OXIDATION-REDUCTION POTENTIAL

The results of previous experiments indicate that the presence of methylene blue accelerates the potential change in cream and skim milk when these solutions are exposed to sunlight. In order to study more fully the rôle played by methylene blue in this reaction, skim milk containing 1 per cent sodium oleate was divided into six parts, and methylene blue was added as follows (1) None, (2) 1:400,000, (3) 1:200,000, (4) 1:100,000, (5) 1:50,000, (6) 1:25,000. The tubes were placed in the water bath and exposed to sunlight. The potential curves and points at which visible reduction was completed are presented in Figure 5.

The potentials of all the samples not only drifted to more negative values when the tubes were exposed to sunlight, but except in the case of sample 6 (1:25,000), the fall of potential was directly related to the concentration of dye. The potential of sample 6 did not reach the negative limits attained by samples 4 (1:100,000) and 5 (1:50,000). The most marked difference observed was between samples 1 (no dye) and 2 (1:400,000). It is quite evident that the presence of only a small quantity of dye greatly accentuates the

potential change induced by sunlight. The accelerating action of the dye is not directly proportional to the amount of dye added. The addition of methylene blue in concentrations higher than 1:200,000 did not materially increase the reducing intensities induced by sunlight. The potentials of all the samples remained fairly constant after the initial drift toward the negative side. After the samples had incubated for five hours the potentials dropped rapidly to more negative limits. These latter changes in potential are due, no doubt, to bacterial activity.

In Figure 5 it may be observed that the final negative limits attained by the samples, with one exception, are inversely related to the concentration of dye added. The exception noted is sample 1, the negative limit of which is slightly more positive than that of sample 2. The time required for visible reduction in the six samples

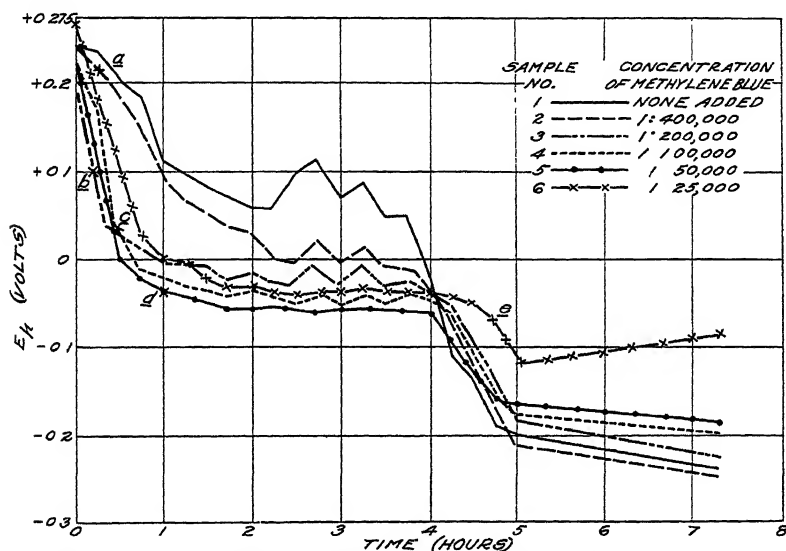


FIGURE 5—Effect of sunlight on the potential time curves of skim milk containing various concentrations of methylene blue when exposed to sunlight, complete reduction took place at points *a* to *e*

increased directly with the amount of dye added. The time varied from 15 minutes for sample 2 (1:400,000) to 285 minutes for sample 6 (1:25,000). The  $E_h$  values at which visible reduction of the dye was complete (*a*, *b*, and *c*) became more negative with each increase in concentration. Sample 6 was not completely reduced by the sunlight. Though lighter in shade, some color was still discernible at the time sunlight was no longer available. Decolorization of this sample was effected only after bacterial action had induced a more negative reducing intensity.

#### EFFECT OF ALTERNATE LIGHT AND DARKNESS ON THE OXIDATION-REDUCTION POTENTIAL

Figure 6 illustrates the change in oxidation-reduction potentials induced by alternately placing a solution in the light and in the dark.

Samples of skim milk and market milk were each divided into two portions and methylene blue (1:200,000) added to one of each.

These four samples were exposed to sunlight, and when the potential of a sample had drifted toward more negative values the sample was covered with a sleeve of heavy black paper to exclude the light. The effect on the potential of alternately placing milk or skim milk in the light and dark is illustrated in Figure 6. At the points on the curves labeled *d* the samples were placed in the dark, and at points *l* they were again exposed to light. The potential of the sample of skim milk plus methylene blue dropped quickly to  $E_h + 0.08$  volt at the beginning of the experiment, when the sample was placed in the dark the potential rapidly returned to the more positive  $E_h$  value of  $+0.2$  volt. When the sample was again placed in the light the potential drifted quickly back to an  $E_h$  value of approximately  $+0.1$  volt. After the sample had been exposed to three hours of incubation, the sun, although still shining, had disappeared behind adjacent buildings, thereby diminishing the intensity of the effective light. Skim milk

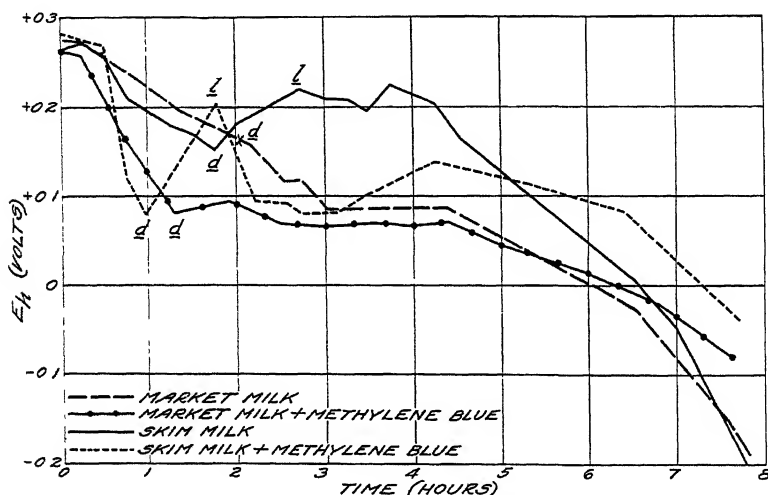


FIGURE 6.—Potential-time curves of market and of skim milk, with and without the addition of methylene blue, when placed alternately in sunlight and in the dark, the samples were placed in the dark at points indicated by *d* and in sunlight at points indicated by *l*.

containing methylene blue (1:200,000) was consistently found to be very responsive to any diminution of light intensity, as is evidenced by the slight drift in potential between the third and fourth hour of experiment. Effective sunlight was no longer available after the fourth hour of this particular experiment.

The potential curve of skim milk without methylene blue shows that alternate placing of the sample in the light and in the dark affects the potential drift. However, the response of the electrode potential to light is not as great as in the case of skim milk plus dye.

The potential curves of market milk with and without dye show that the potentials drift to more negative values when exposed to sunlight, but do not return to more positive values when placed in the dark. The sample of market milk when placed in the dark not only failed to respond by swinging to more positive values, but continued its uninterrupted negative potential drift.

These observations suggest (1) That methylene blue accentuates the response of the electrode potential to the effects produced by the

presence or absence of light, and (2) the presence of fat has a deterring influence on the potential drift as induced by light. This latter observation is in harmony with those made in connection with Figures 1 and 2.

The complexity of the oxidation-reduction system or systems in milk and cream renders hazardous any attempt to speculate on the probable mechanism of light in these observations. The trend toward more negative potentials when milk is exposed to light suggests, however, the participation of some ingredient which is temporarily oxidized while under the influence of light, and which regains its electron at the expense of the electromotively active system as soon as the effect of the light is removed.

It is possible that the deterring influence of fat on the reversibility of this process may be due in part to the sluggishness of electrodes in the presence of increasing amounts of fat. However, if the effect were entirely apparent rather than real—that is, if increasing percentages of fat affected only the electrode sensitivity—one would expect a return of the blue color when previously exposed cream was returned to the dark. Since the failure of the electrode to record a trend toward positive values is corroborated by a failure of the blue color to return when cream which has been exposed to the light is returned to the dark, it suggests that the fat exerts an actual deterring influence on the reversibility of the reaction. Whether it serves as a shield or protector of the electromotively active oxidation-reduction system or actually participates in the sharing of electrons can not be deduced from the observations at hand.

#### EFFECT OF ARTIFICIAL LIGHT ON THE OXIDATION-REDUCTION POTENTIAL

It is a common practice in the determination of quality of milk by the methylene blue reduction test to incubate the samples in a constant temperature incubator. The temperature of such incubators is usually regulated by using electric lights as a source of heat. It has been observed that when samples are incubated in this manner those nearest the light are reduced in the least time.

In a preliminary experiment samples near an electric light were reduced 2.5 hours earlier than shielded samples. In order to determine the cause of this difference in reduction time the following experiment was conducted. A sample of market milk of good quality was divided into four parts and the standard amount of methylene blue was added. The samples were placed in an incubator maintained at 37° C. by two 75-watt bulbs. One of these burned constantly and the other was operated intermittently by the thermostat. Duplicate tubes of milk were protected from the light by sleeves of heavy black paper and two others were exposed to the light rays from the electric bulbs. Representative potential curves and reduction times of two of these are presented in Figure 7.

An examination of these curves shows that artificial light affected the potentials and reduction time in much the same manner as was observed in the case of sunlight. The potential of the exposed sample drifted slowly toward the negative side, whereas that of the shielded sample remained fairly constant for six hours. As shown in Figure 7, the exposed sample was completely reduced about 2.5 hours sooner than the shielded sample.

Temperature was not a factor in hastening reduction of the exposed samples. Since the temperature of the exposed samples was  $1^{\circ}\text{C}$  lower than that of the ones in the dark, it is not possible to attribute the more rapid reduction to this factor.

### SUMMARY

The potentials of cream, whole milk, and skim milk drifted toward the negative side when these solutions were exposed to sunlight. Potential changes to both more positive and more negative values were deterred by the presence of fat. This influence exerted by fat was especially noticeable when solutions were alternately placed in the sunlight and in the dark. The addition of fat to skim milk hastened the reduction time of methylene blue in samples exposed to the sunlight, owing probably to the tendency of fat to elevate the zone of reduction. Sodium oleate and sodium stearate also shortened the reduction time, but did so by causing a more rapid fall of potential.

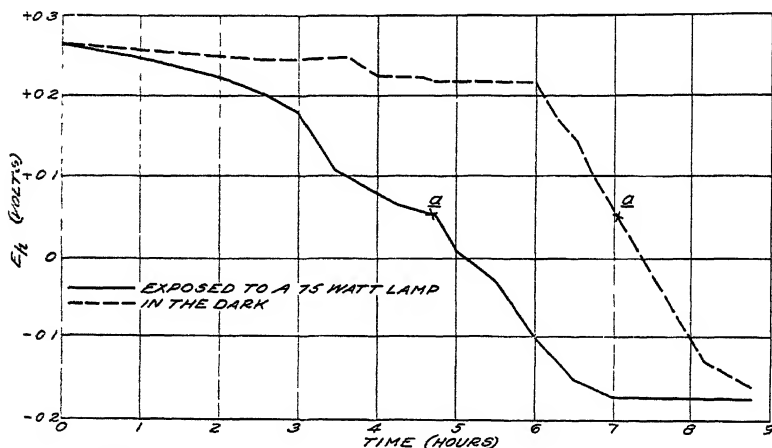


FIGURE 7 — Effect of exposure to a 75-watt electric light upon the potential time curves and reduction time of market milk, complete reduction took place at point *a*

The addition of methylene blue to skim milk or cream accentuated the potential changes induced by sunlight. With each increase up to 1:25,000 in the concentration of dye added to skim milk containing sodium oleate, the reducing intensities induced by sunlight were progressively more negative.

Visible reduction of methylene blue induced either by sunlight or bacterial activity took place within the  $E_h$  limits characteristic for the particular sample.

The reducing intensity induced by bacterial activity was more negative than that induced by sunlight. In the case of sunlight the negative limits reached were seldom below zero, as compared with a reducing intensity of  $-0.2$  volt induced by bacteria.

These observations confirm Whitehead's conclusion, that reduction of methylene blue by light is a reaction distinct from the reaction induced by bacteria.

It was observed that as the solution developed a progressively more negative potential the methylene blue decolorized whenever this potential passed through the zone of reduction characteristic of

this dye Similarly, the blue color reappeared when the solution developed a potential sufficiently positive to oxidize the dye present When skim milk plus methylene blue which had been reduced by sunlight was placed in the dark, the potentials quickly became sufficiently positive to oxidize the dye

Artificial light hastened the reduction of methylene blue in market milk Light from a 75-watt electric lamp induced a potential drift in milk which differed only in degree from that observed in the case of sunlight The reduction of methylene blue in one sample of milk was hastened 2.5 hours by exposure to light from an electric bulb

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## CHARACTERISTICS OF DISPERSABLE ORGANIC COLLOIDS IN PEATS<sup>1</sup>

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### INTRODUCTION

Although the importance of fine organic matter in soil is generally recognized, studies of soil colloids have dealt mainly with inorganic constituents. In a previous report (16)<sup>3</sup> some evidence of high base-exchange capacity of peat soils was noted. This characteristic has recently been reported by several investigators (2, 9, 10, 11, 13). There is also evidence that organic colloidal matter in soils possesses a large capacity for absorbing moisture (14).

The experiments herein reported were undertaken to determine the amount and composition of colloids in several peat-profile layers, to learn how base-exchange capacity changes during decomposition of organic materials, and to obtain information as to the nature and possible means of increasing or conserving the base-exchange capacity of soils.

### PREVIOUS WORK

The base-exchange property of soils was reported by Way in 1850 (30). It was recognized by Gedroiz (7) and by Odén (14) that organic as well as inorganic colloidal matter in soils may contribute to their base-exchange values. In an earlier study the writer (17) found evidence of a large base capacity in peat soils. However, in the more acid peats, a large part of the total base-exchange capacity was due to exchangeable hydrogen. Soon after the publication of this report a study by Hissink (9) with some 13 peaty soils came to the writer's attention. His conclusions supported this view. Alben (1) reported finding the base-exchange capacity of peat to be some seven times that of normal soil. Bayer (2) studied the effect of removing organic matter from 4 soils by hydrogen-peroxide treatment, and concluded that from 30 to 60 per cent of their base-exchange capacity was due to organic matter. McGeorge (11) worked with a score of soils, most of which were of a peaty character, and obtained evidence that high base-exchange capacity was related to the lignin content.

Recently Smolik (24) reported base replacement far greater for soils rich in organic matter and markedly reduced by treatment with

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 110.

hydrogen peroxide Odén (14) about a decade ago reported that humic-acid forms a salt with ammonia and that liming results in the formation of a calcium humate which he regarded as an excellent regulator or buffer for the prevention of a strong acid reaction It was of low solubility and difficult to wash out Demolon and Barbier (3) were also led by their experiments to suggest that the clay and humate fractions form a complex, and that this argillo-humate complex is influenced by the cations absorbed Doughty (4) studied phosphate fixation in peat, and concluded that calcium, iron, or aluminum when present in soluble form cause precipitation of phosphate in peat Similar results were obtained by Marshall (12), who reported that calcium humate exercises a protective action and sensitizes the soil colloid Dunnewald (5) noted the relation of calcium carbonate to soil organic-matter content and vegetation Recently Mattson (13) reported investigations of base capacity of iron and aluminum humates when in different proportions and also at different pH values High humic content in association with an alkaline reaction seems to possess large base-absorbing capacity

A review of the literature indicates the need of further information in regard to the characteristics of natural organic soil colloids in order that methods may be developed of increasing and maintaining high base-exchange capacity in soils In the present study natural organic soil colloids from several peat profiles have been isolated and studied, perhaps for the first time

## EXPERIMENTS WITH PEAT COLLOIDS

### DESCRIPTION OF PEAT-PROFILE SAMPLES

Profile samples of representative peat formations from widely separated sections were secured for the present study and are briefly described as follows

- 1 Saw-grass peat from the Brown plantation, Belle Glade, Fla., described in a previous publication of this department (6)
- 2 Saw-grass peat, fresh sample, taken in slightly shallower peat, about one-half mile distant from No 1
- 3 Virgin willow-sedge peat from Lake Labish, near Salem, Oreg
  - 0 to 6 inches.....Dark-brown finely fibrous woody-sedge peat
  - 6 to 48 inches.....Brown fibrous peat of sedge and soft wood
  - 48 to 60 inches.....Fibrous peat with some sedimentary materials, diatoms, sponge spicules, spores, and millet fragments
4. Similar to No 3 and about one-fourth mile distant Cropped about 20 years
- 5 Sphagnum peat from Cottage Lake, near Seattle, Wash
  - 0 to 2 inches.....Gray-brown sphagnum moss with related vegetation, such as swamp laurel, wild cranberry, and rhododendron
  - 2 to 10 inches.....Raw yellow-brown sphagnum peat
  - 12 to 24 inches.....Brown fibrous sphagnum peat
  - 24 to 36 inches.....Dark-brown fibrous peat, partly decomposed
6. Clackamas peaty gravelly loam, or "loose land" from West Stayton, near Salem, Oreg
  - 0 to 8 inches.....Brown sooty gravelly loam
  - 18 to 30 inches.....Yellowish-brown gravelly loam
7. Sedimentary tule-sedge peat, Lower Klamath Marsh, Oreg
  - 0 to 14 inches.....Dark-brown fibrous tule-sedge peat with some carbonized material
  - 14 to 28 inches.....Gray-brown finely fibrous peat
  - 28 to 42 inches.....Gray sedimentary muck with diatomaceous material

- 8 Sedimentary peat, Orleans County, N Y, collected and described by A P Dachnowski-Stokes A typical profile of peat areas in the western Ontario glacial-lake plain
- |                      |                  |
|----------------------|------------------|
| 0 to 6 inches-----   | Woody peat       |
| 12 to 18 inches----- | Sedimentary peat |
| 30 to 36 inches----- | Reed peat        |
| 60 to 72 inches----- | Sedimentary peat |

#### EXPERIMENTAL METHODS

The method of separation of organic colloid was similar to that described by Robinson and Holmes (21) with modifications, as noted later Absorption tests were made according to the method of Robinson (19), and the general inorganic analytical procedure followed was that of Robinson (18) Base-exchange capacity was determined by treating with one-twentieth normal hydrochloric acid, then saturating with normal neutral barium chloride, and, after washing out the excess of saturant with warm distilled water till free of chlorides, displacing with normal ammonium chloride The barium absorbed was determined gravimetrically as barium sulphate

Organic analyses were made according to the method of Waksman and Stevens (29) as modified by Feustel and Byers (6) Lignin and hemicellulose were obtained from flax shives and sphagnum moss by the method of Phillips (15)

#### PRELIMINARY EXPERIMENTS

Some preliminary tests were made to determine the most suitable procedure for separating the colloid from peat samples These included the use of different amounts of sample, removal of bases, hand kneading, electric agitation (21), mechanical shaking, agitating by means of a Bouyoucos shaker, addition of sodium oxalate, and previous extraction with ether The effects of amount of dilution (22), rate of centrifuging, and yield and character of different fractions were also considered.

From these tests it was found advisable to use sufficient fresh moist peat to yield from 100 to 200 gm of material that would pass through a 2-mm sieve Shaking the sample overnight in a 1-gallon sirup bottle two-thirds full of distilled water, with a slight addition of sodium oxalate (approximately 0.1 gm), proved very helpful The shaker used for preparing samples for mechanical analysis was fitted with a drawer to hold two 1-gallon bottles The electric agitator was used before each run of the supercentrifuge, and material coarser than 2 mm was omitted after the second run Eight runs were made, in which a total of 150 to 200 liters of water was used, according to the profile dealt with Running the peat suspension through the centrifuge at the rate of 1 liter in 5 seconds separated a colloid fraction mainly below  $1\mu$ , with a few aggregates in excess of  $2\mu$  in diameter Satisfactory yields of colloid were thus secured

This colloidal fraction, reduced by a battery of Pasteur-Chamberland filters to slightly less than 2 quarts and homogenized by passing through a fine Gooch crucible, carried sufficient solids to give a 2 or 3 gm sample from 200 cc of suspension Most of the samples employed for base capacity and analyses weighed approximately 2 gm.

The bowl fraction and even material between 1 and 2 mm in diameter were found to manifest somewhat colloidal properties.

## AMOUNT AND ABSORPTIVENESS OF COLLOIDS FROM PEAT

The amount (yield) and absorptiveness (character) of peat colloids are shown in Table 1

TABLE 1—Amount and absorptiveness of peat colloids

Sample No	Location	Description of sample	Depth	pH	Dry weight of sample	Material greater than 2 mm in diameter	Material less than 2 mm in diameter	Weight of dry colloid extracted	Colloid present in 2-mm fraction	Ash in colloid	Absorption of water over 33 per cent H <sub>2</sub> SO <sub>4</sub>
1	Belle Glade, Fla	Saw-grass peat	In		Gms	Gms	Gms	Gms	P ct	P ct.	P ct
			0-4	5.3	94.75	14.1	80.65	2.886	3.05	18.28	-----
			4-6	6.2	26.79	4.1	22.71	1.432	3.34	18.60	-----
			32	6.3	29.21	2.3	26.95	1.833	6.62	26.66	-----
			49	6.3	22.40	5.2	17.16	2.088	9.32	-----	-----
2	Do	do	63	6.7	21.19	8	20.43	3.075	14.51	-----	-----
			94-96	7.1	20.60	3.1	17.54	1.980	9.61	21.70	-----
			0-4	4.6	236.10	133.0	113.10	20.100	25.70	25.70	46.86
			4-6	5.2	142.40	32.0	110.40	26.600	24.10	24.10	47.13
			32	6.6	127.70	15.0	114.70	45.100	39.30	39.30	43.61
3	Salem, Oreg.	Virgin willow-sedge peat	49	6.6	115.80	37.0	78.80	20.860	26.40	26.40	43.86
			63	6.8	104.60	31.0	73.60	22.000	29.90	29.90	43.66
			86-88	7.2	114.70	32.0	82.70	46.800	56.50	56.50	39.50
			0-6	6.5	256.80	10.2	246.00	54.970	22.34	62.59	53.84
			12-42	6.5	116.20	3.8	112.40	55.130	31.25	61.49	35.56
4	Do	Willow-sedge peat (cropped 20 years)	48-60	6.0	111.30	2.8	108.50	56.740	52.30	-----	46.07
			0-6	6.4	240.00	8.4	231.60	32.040	13.83	48.21	59.23
			12-42	6.5	102.90	2.5	100.70	26.440	26.25	28.96	38.52
			48-60	7.1	102.30	1.5	100.80	22.780	22.59	32.94	35.77
5	Seattle, Wash.	Sphagnum peat	2-10	5.1	652.00	300.0	352.00	18.500	5.28	19.59	45.57
			12-24	6.4	80.60	40.0	40.60	2.178	5.35	16.66	46.51
			24-36	6.4	311.00	200.0	111.00	14.490	13.00	11.71	42.16
			0-8	5.7	142.00	50.0	1,092.00	115.400	10.57	53.32	37.24
			18-30	6.0	93.00	40.0	530.00	26.100	4.92	66.48	29.23
7	Lower Klamath Marsh, Oreg	Sedimentary tule-sedge peat	0-14	7.1	176.00	1.0	177.00	53.28	30.10	83.19	34.74
			14-28	7.0	181.00	0	181.00	18.28	10.10	63.83	33.58
			28-42	6.2	186.60	0	186.60	8.14	4.35	56.77	50.83
			0-6	5.8	164.00	25.0	111.00	11.60	9.56	53.87	42.01
			12-18	5.8	121.00	15.0	106.00	16.65	15.71	18.92	46.04
8	Orleans County, N Y	Semimentary peat	30-36	5.7	80.00	1.0	79.00	11.74	14.86	13.04	45.79
			60-72	7.4	60.60	1	60.10	9.18	15.30	20.37	50.16

\* See U S Dept Agr Tech Bul 214 (6), for pH data for Florida peat

The peat samples ranged from neutral to distinctly acid in character. Moist samples weighing from 200 to 1,000 gm were used, the initial moisture content ranging from 100 to 800 per cent. Considerable difference was shown in the content of coarse material in the different layers of a single profile. The sedimentary layers were low in ash and yielded relatively large percentages of organic colloid. This colloid yield ranged from 3.05 to more than 56.5 per cent of the sample passing a 2-mm. sieve, expressed on a dry-weight basis.

Absorption of moisture over 3.3 per cent sulphuric acid was determined with samples which had been slowly dried on the edge of the steam bath and ground to pass through 130-mesh bolting cloth. The average absorption of peat colloid was 45 per cent as compared to about 30 per cent for inorganic soil colloid. The values obtained ranged from approximately 29 to 50 per cent. The lower values are for colloids having large ash content.

## PROXIMATE ORGANIC COMPOSITION AND BASE-EXCHANGE CAPACITY OF PEAT COLLOIDS

Organic analyses were made of the different layers in peat profiles. These are summarized in Table 2.

TABLE 2—Proximate organic composition of peat colloids

[In per cent of colloid]

Sample No	Location	Description of sample	Depth	Dry weight of colloid used	Ether-soluble material in colloid	Alcohol-soluble material in colloid	Hot-water soluble material in colloid	
							Dry matter	Ash
			Inches	Grams	Per cent	Per cent	Per cent	Per cent
1	Belle Glade, Fla.	Saw-grass peat	0-4	3 88	0 206	0 42	4 81	0 87
			4-6	3 04	336	46	2 78	68
			32	4 11	224	27	1 20	39
			49	2 98	269	58	1 53	57
			63	2 20	432	61	1 55	72
3	Salem, Oreg.	Virgin willow-sedge peat	86-88	3 13	517	58	1 43	55
			0-6	5 99	250	86	3 33	56
			12-42	4 29	110	67	1 79	35
4	Do.	Willow-sedge peat (cropped 20 years)	48-60	6 94	870	50	1 22	22
			0-6	3 10	340	50	3 07	37
5	Seattle, Wash.	Sphagnum peat	12-42	3 46	220	87	1 96	42
			48-60	3 33	420	71	1 63	47
			2-10	1 48	550	4 30	7 79	-----
6	Salem, Oreg.	Clackamas peat, gravelly loam (loose lan 1)	12-24	1 30	600	5 33	7 43	-----
			24-36	1 11	1 160	6 23	7 29	-----
			0-8	9 23	130	57	1 15	-----
8	Orleans County, N. Y.	Sedimentary peat	18-30	5 22	140	38	1 00	-----
			0-6	1 40	1 250	2 16	5 75	1 10
			12-18	1 48	540	2 12	6 21	1 28
			30-36	1 61	600	1 22	3 65	58
			60-72	1 08	910	2 08	5 00	1 00

Sample No	Location	Hemicellulose	Cellulose	Lignin			Base-exchange capacity per gram of colloid	Total nitrogen	
				Gross lignin	Ash	Ash-free lignin*		In whole peat	In colloid fraction
		Per cent	Per cent	Per cent	Per cent	Per cent	Milhequivalent	Per cent	Per cent
1	Belle Glade, Fla.	3 78	0 73	44 25	0 77	43 48	0 695	3 58	3 54
		6 24	92	51 30	91	50 39	7 11	3 02	3 54
		2 53	1 32	75 22	72	74 59	870	3 14	3 58
		1 53	3 26	74 91	65	74 26	664	2 63	3 16
		3 23	3 11	72 75	83	71 92	729	2 81	3 35
3	Salem, Oreg.	5 61	95	59 92	2 06	56 96	605	2 09	1 81
		4 17	2 08	55 75	29 42	26 33	338	2 43	5 66
		3 98	1 93	54 06	22 35	31 71	384	2 01	4 02
		1 83	3 05	58 06	34 22	23 84	437	1 83	4 72
		5 93	1 49	49 73	27 44	22 29	405	2 34	3 22
4	Do.	4 61	2 45	58 25	12 64	45 61	374	2 43	3 52
		4 43	2 22	62 84	12 73	50 11	326	2 48	5 81
		29 91	14 59	39 74	5 67	24 07	850	95	3 00
5	Seattle, Wash.	32 23	10 90	27 03	4 53	22 50	729	1 42	3 83
		29 95	7 66	46 54	5 28	41 26	491	1 56	2 55
		3 41	2 75	34 43	20 01	14 42	165	76	1 70
6	Salem, Oreg.	3 57	4 77	11 73	8 54	3 19	086	32	50
		3 60	2 83	40 44	15 47	24 97	533	2 64	2 02
8	Orleans County, N. Y.	4 86	4 86	54 42	4 29	50 13	613	2 63	2 74
		5 31	5 39	64 83	2 83	62 00	596	2 20	3 51
		8 70	5 00	54 00	5 08	48 92	1 218	2 05	3 81

\* Estimated by difference

The method used seems to yield more satisfactory results with material low in inorganic matter. The hemicellulose content of sphagnum or high-moor peat appears to be relatively large. Of special interest is the so-called lignin content, which constitutes more than half of the sample taken in several cases, when expressed on the ash-free basis. The saw-grass peat carries a large amount of ligneous material, and profile No. 6 is especially well supplied. Old, sedi-

mentary layers of low ash content appear to run high in ligneous material<sup>1</sup>

The base-exchange capacity is expressed in miliequivalents per gram of colloid. There appears to be a tendency for a high base capacity to correlate with high content of ligneous substance, further evidence of this will be presented later. The state of this material as conditioned by the presence of mineral matter and bases may affect this value, as suggested by data such as are shown for the sixth-foot layer of peat sample No. 8.

Total nitrogen was determined for the peat and also for its colloid fraction. The nitrogen content of the colloid is usually higher, and in some soils, as in Nos. 3 and 4, there is approximately twice as much nitrogen in the colloid as in the whole peat. Determinations of ammonia nitrogen in colloid samples from the three layers of profile No. 3 were made by distillation with magnesium oxide to determine whether much exchange ammonia was present. The values obtained for the three layers, beginning with the top one, were 0.065, 0.072, and 0.061 per cent ammonia nitrogen. This is only a small fraction of the total nitrogen and does not account for the concentration of nitrogenous matter in the colloid part of the peat. It appears that two decades of cropping have materially lowered the nitrogen content of willow-sedge peat. This large amount of nitrogen, if available, would enhance the value of colloidal organic matter in relation to soil productivity. The content of nitrogen in sphagnum peat colloid is low compared to that of the sedge, or low-moor, peat colloid.

#### BASE-EXCHANGE CAPACITY OF DIFFERENT PEATY MATERIALS

The amounts of colloid secured from certain soils were not adequate for full analyses, yet the base-exchange capacities were determined and are given in Table 3.

TABLE 3—Base-exchange capacity of various peaty materials

Sample	Description of sample	Depth	Description of material	Base exchange capacity per gram of colloid
		<i>Inches</i>		<i>Miliequivalents</i>
A	Saw-grass peat from profile No. 1	0-4	Natural peat.....	0.378
			Fraction 1 to 2 mm diameter wet...	361
			Fraction 1 to 2 mm diameter dry...	293
			Fraction 1 to 2 mm diameter dry, ground	376
Do.	do.	4-6	(Centrifuge bowl fraction).....	509
		0-4	do.....	664
		4-6	do.....	464
B	Colloids in saw-grass peat from profile No. 1	32		950
		49		1,019
		63		169
		96		558
		0-1½		962
C	Colloids in tule-sedge peat, Klamath, Oreg.	14-28		265
		28-42		127
D	Dopplerte* from the Netherlands			255
				736

\* Supplied by A. P. Dachnowski-Stokes

The base-exchange capacity appears to increase with fineness of natural sample and with grinding, and to be reduced by drying. The highest base-exchange value for saw-grass peat colloid was obtained from the sedimentary layer at a depth of 32 inches. Colloid from Klamath peat contains a large portion of diatomaceous (siliceous) matter and is of only medium base capacity. The layer of saw-grass peat taken at a depth of 32 inches is sedimentary. The high base capacity obtained for such mucklike material may be due in part to molecular concentration at the interfaces of the mixture. The indicated tendency is for dilution with sand to increase the base capacity per gram of colloid.

The dopplerite is found in veins or layers in peat, where it has presumably accumulated over a long period of time (27). The sample was jet black, highly colloidal, and had a moisture content of 688 per cent when received. The base-exchange capacity for this substance is large.

## EXPERIMENTS WITH COMPOSTED MATERIALS

### LOSS OF DRY ORGANIC MATTER FROM COMPOSTED PLANT MATERIALS

In order to provide material for the study of base-exchange capacity of decaying organic matter at different stages of decomposition, several dozen jars were arranged for composting in the soil bacteriology greenhouse at Arlington Experiment Farm, Rosslyn, Va. Duplicate series of stoneware jars were employed for each of the organic materials used. These materials were sweetclover straw, flax shives, sphagnum moss, sphagnum peat, and saw-grass peat. Chemical or other treatments made to ascertain their effect on decomposition and base-exchange capacity are indicated in Tables 4 and 5. Observations were made periodically to determine rate of carbon-dioxide evolution, temperature reaction, and moisture content. Watering, except for water-logged jars, was usually just sufficient to maintain a little free liquid which was drained out by decantation weekly and returned to the top of the material in the jar. Carbon-dioxide measurements were for one pair of jars of each series, for only one hour a week, and by the method of Humfeld (10).

The loss in organic materials is shown in Table 4.

TABLE 4 — *Loss in organic materials composted four months*

[Loss on dry-matter basis]

Treatment	Sweetclover straw		Flax shives		Sphagnum peat		Sphagnum moss	
	Loss	Residue	Loss	Residue	Loss	Residue	Loss	Residue
	<i>Per cent</i>	<i>pH</i>	<i>Per cent</i>	<i>pH</i>	<i>Per cent</i>	<i>pH</i>	<i>Per cent</i>	<i>pH</i>
Distilled water.....	53.9	7.7	11.3	5.9	0.0	4.2	8.0	4.8
Soil and manure infusion.....	50.4	7.8	11.5	6.0	6.4	4.2	8.5	4.3
3 per cent $\text{CaCO}_3$ .....	54.0	8.0	12.6	6.6	3.1	5.2	9.0	4.8
3 per cent $\text{CaCO}_3$ and 2 per cent $\text{NH}_4\text{NO}_3$ .....	55.7	8.2	27.0	6.7	10.9	5.6	-----	-----
3 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$ .....	57.5	8.0	29.5	7.1	12.0	4.5	18.2	4.7
2 per cent $\text{NH}_4\text{NO}_3$ and 2 per cent $\text{K}_2\text{HPO}_4$ <sup>b</sup> .....	48.3	8.1	28.7	5.8	10.6	4.8	-----	-----
3 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$ (water-logged).....	47.5	8.1	25.3	7.2	10.0	4.2	14.0	4.6

<sup>a</sup> 10 per cent sweetclover added instead of  $\text{CaCO}_3$

<sup>b</sup> 3 per cent  $\text{CaCO}_3$  added at end of 60 days

The sweetclover straw underwent a flash decomposition during the first six weeks, as evidenced by temperature rise and carbon dioxide evolved. Thermograph records show that the mean temperature of the greenhouse air was approximately 24° C. During the first five weeks sweetclover composts ran temperature 4° or 5° above that of the air. Insulation of two flax jars to conserve heat of reaction appeared to aid decomposition. The sweetclover composts lost from 47 to 60 per cent of the original organic matter in four months. Decomposition of other materials was slow and ranged from 6 to 30 per cent. The flax had already undergone flash decomposition in retting and was also only. The acid sphagnum peat, and the saw-grass peat also, proved to be resistant to decay.

The sphagnum moss seemed to undergo more active decomposition than did the older sphagnum peat. Sphagnum decomposition appears to have been aided by nitrate additions.

TABLE 5—Base-exchange (milliequivalents) capacity of plant materials and composts

Material	Treatment	Base-exchange capacity per gram of material		
		Fresh material	Material composted 2 months	Material composted 4 months
Sweetclover straw (3-gallon jars)	None.....	0 125		
	Soil infusion.....		0 211	0 168
	5 per cent CaCO <sub>3</sub> .....		188	323
	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		212	291
	2 per cent NH <sub>4</sub> NO <sub>3</sub> and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		154	223
	Distilled water only.....		217	202
Sphagnum moss, 0-2 inches (2-gallon jars)	None.....	490		
	Soil infusion.....		528	669
	10 per cent of sweetclover added.....		508	596
	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		495	670
	Same as above, with high water table.....		507	615
	Distilled water.....		451	589
Sphagnum moss, 2-10 inches (2-gallon jars)	None.....	570		
	Soil infusion.....		559	631
	5 per cent CaCO <sub>3</sub> .....		504	591
	5 per cent CaCO <sub>3</sub> and 2 per cent NH <sub>4</sub> NO <sub>3</sub> .....		492	576
	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		520	588
	2 per cent NH <sub>4</sub> NO <sub>3</sub> and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		497	619
Flax shives (1-gallon jars)	Distilled water.....		500	662
	None.....	067		
	Soil infusion.....		073	168
	5 per cent CaCO <sub>3</sub> .....		094	185
	5 per cent CaCO <sub>3</sub> and 2 per cent NH <sub>4</sub> NO <sub>3</sub> .....		086	161
	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		090	164
Saw-grass peat, 0-4 inches (1-gallon jars)	2 per cent NH <sub>4</sub> NO <sub>3</sub> and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		080	214
	Distilled water.....		073	139
	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....			
	None.....	695		
	Soil infusion.....		598	689
	5 per cent CaCO <sub>3</sub> and 2 per cent NH <sub>4</sub> NO <sub>3</sub> .....		565	581
Wheat straw (350-pound lots)	5 per cent CaCO <sub>3</sub> , 2 per cent NH <sub>4</sub> NO <sub>3</sub> , and 2 per cent K <sub>2</sub> HPO <sub>4</sub> .....		577	667
	Distilled water.....		567	677
	None.....	209	(c)	
	Water.....		219	
	300 pounds acid peat added.....		193	
	Chopped, 14 pounds calurea containing 37 per cent N added.....		206	
	Long, 14 pounds calurea added.....		202	
	Chopped, 42 pounds dried blood added.....		230	
	Long, 42 pounds dried blood added.....		168	

\* 5 per cent CaCO<sub>3</sub> added at end of 60 days

<sup>b</sup> Jars insulated for control of heat

<sup>c</sup> Composted six weeks

Four 1-gallon composts of saw-grass peat were provided for each of three layer samples. This material decays very slowly. Neither inoculation nor drainage gave significant results. Addition of nutrients appeared to increase the rate of decomposition during the first two months. Decomposition was most active with soil from the layer 0 to 4 inches from the surface, and losses of dry organic matter up to approximately 20 per cent were indicated. The material from the layer between depths of 4 and 6 inches lost about half as much as the fresher surface material, while the older material from the layer at a depth of 32 inches gave values that were scarcely significant.

Composite samples were taken at the end of two months and again after four months of composting. These were weighed, dried, ground, and subjected to base-capacity tests as summarized in Table 5.

The initial plant materials used in composts were found to manifest base-exchange capacities in different degrees, which might be of importance in choosing material for green manure or stable litter. Base-exchange capacity appears to increase during decomposition. Chemicals that aid decomposition seem to favor increase in base capacity, especially during the first half of the decomposition period. They may affect reaction, aid formation of additional products, or affect the physical state of the system.

Samples of composts of wheat straw prepared for mushroom growing were supplied by Edmund B. Lambert, of the Bureau of Plant Industry, who suggested that chopping helps in decomposition by aiding compaction or perhaps by exposing cut ends to attacks of decomposition microorganisms. Base-exchange capacity tests indicate that chopping is of value in promoting decomposition.

A study of these materials and certain of the compost residues is reported in Table 6.

TABLE 6—Proximate composition (per cent) of plant materials and compost residues <sup>a</sup>

Material	Treatment	Ether soluble	Alcohol soluble	Hot-water soluble	Hot-water soluble ash	Hemicellulose	Cellulose	Lignin	Lignin ash
Sweetclover straw	None, dry	0.90	4.89	8.96	1.56	8.26	18.67	15.68	0.51
	Distilled water	1.02	3.60	7.17	1.93	14.74	17.10	26.98	1.07
	5 per cent $\text{CaCO}_3$	4.6	3.12	8.06	3.0	10.62	3.94	43.98	5.69
	5 per cent $\text{CaCO}_3$ and $\text{NH}_4\text{NO}_3$	1.36	3.31	9.43	2.57	11.27	10.61	31.32	2.97
	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$	87	2.78	12.14	4.92	9.92	10.23	25.82	6.35
Flax shives	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$ , colloidal fraction	45	2.05	21.52	6.34	6.26	11.87	27.76	6.35
	None, dry	69	2.66	3.38	30	8.31	24.20	25.05	.43
	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$	1.09	2.10	6.38	1.68	23.94	5.17	51.47	1.18
Sphagnum moss, 0-2 inch layer	None, dry	1.62	3.22	4.54	43	12.94	13.61	18.77	1.28
	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$	42	5.43	6.96	2.07	20.20	6.07	44.39	2.65
	None, dry	72	3.14	4.87	06	11.11	9.50	22.51	1.69
Sphagnum peat, 2-10 inch layer	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$	1.33	5.58	7.14	2.40	23.31	13.27	28.25	1.20
	None, dry	76	2.25	7.16	1.92	7.78	5.50	43.91	1.61
	5 per cent $\text{CaCO}_3$ , 2 per cent $\text{NH}_4\text{NO}_3$ , and 2 per cent $\text{K}_2\text{HPO}_4$ , with drainage	1.58	4.23	7.75	2.71	25.94	3.42	50.57	3.09
Wheat straw	None, dry	1.36	4.90	7.21	-----	25.89	38.97	13.33	1.18

<sup>a</sup> Composted 4 months

## PROXIMATE ORGANIC COMPOSITION OF PLANT MATERIALS AND COMPOSTS

Organic analyses were made of plant materials used in composts as well as of numerous composted materials. The results are presented in Table 6. The decrease in more soluble and less resistant constituents is partly masked by additions of soluble nutrients. Except in the case of certain saw-grass peat composts, drainage was not provided. The most significant change in composition is the concentration of ligneous material. This tends to parallel the increase in base-exchange capacity as decomposition progresses (Table 5).

Determinations of total nitrogen were made for several samples. Threshed sweetclover straw was found to contain 1.223 per cent nitrogen. After the sweetclover had been composted with distilled water for four months the nitrogen content of the residue was found to be 2.44 per cent. No loss of nitrogen is indicated, as the residual dry organic matter was 47.8 per cent of the initial sample. Sphagnum moss contained 1.68 per cent nitrogen, and after being composted with distilled water for four months the content of the residue, as determined, was still 1.68 per cent nitrogen, indicating a loss of nitrogen proportional to dry matter.

## EXPERIMENTS WITH FRACTIONATED SUBSTANCES

## STUDIES WITH CERTAIN FRACTIONS OF ORGANIC MATERIALS

Part of the ligneous fraction separated during the organic analysis of the saw-grass peat profile, sample No. 2, was used for testing its base capacity and was then subjected to repeated treatments with 30 per cent hydrogen peroxide (20). The base-capacity test was repeated on the residue which was later ashed. The results secured are given in Table 7.

TABLE 7—Base-exchange capacity of ligneous fractions of peat, a lignin from sample No. 2, Belle Glade peat colloids

Depth of soil layer	Lignin fraction in colloid	Lignin ash in colloid	Base-exchange capacity of lignin			Dry residue after $H_2O_2$ treatment of colloid	Ashed residue after $H_2O_2$ treatment of colloid	Ash-free residue in colloid
			Per gram of colloid	Per gram of lignin	Residue, after $H_2O_2$ treatment per gram of colloid			
	Per cent	Per cent	Milliequivalent	Milliequivalent	Milliequivalent	Per cent	Per cent	Per cent
0-4 inches	44.25	0.771	0.080	0.181	0.016	3.29	2.46	2.83
4-6 inches	51.30	.908	.063	.123	.014	1.77	1.19	.58
32 inches	75.22	.722	.090	.120	.043	10.52	1.43	9.09
49 inches	74.91	.647	.115	.154	.045	10.43	4.01	6.42
63 inches	72.75	.832	.063	.103	.021	3.01	1.97	1.04
86-88 inches	59.92	2.962	.146	.241	.046	14.94	7.45	7.49

The base-exchange capacity of this ligneous material insoluble in various solvents appears to be larger than that of the residual inorganic soil colloids, yet it is less than was found in the organic soil colloid before fractionation. The extractions may destroy some organic oxide having base-exchange properties. Treatment with hydrogen peroxide removed most of the organic part of the ligneous fraction and destroyed most of its base-exchange capacity.

Similar tests with the ligneous fraction isolated from the willow-sedge peat colloid which had high base-exchange capacity further indicate that the presence of some inorganic material results in increased base-exchange capacity of ligneous material

Tests of base-exchange capacity were made with ligneous material secured in the course of analyses of plant materials, as shown in Table 8. It appears from these tests that this isolated ligneous material does not necessarily possess as large a base-exchange capacity as the material from which it is derived

TABLE 8—Base-exchange capacity of organic materials and of ligneous fractions separated from them

Material	Base-exchange capacity per gram of plant material	Base-exchange capacity per gram of ligneous fraction	Ash in ligneous fraction
	Milli-equivalent	Milli-equivalent	Per cent
Sweetclover straw.....	0 125	0 141	3 26
Sphagnum moss, 0-2 inches.....	490	235	6 51
Sphagnum peat, 2-10 inches.....	570	459	7 51
Flax shives.....	667	131	1 69

#### BASE-EXCHANGE STUDIES WITH PLANT LIGNIN AND LIGNO-HEMICELLULOSE

Samples high in lignin and in hemicellulose were prepared from both sphagnum moss and flax shives by the method of Phillips (15). After the different extractions the hemicellulose was precipitated and washed with alcohol and the lignin boiled with hydrochloric acid to destroy any hemicellulose present, then washed free of chlorides. Samples of oat-hull lignin and corncob lignin were also used for base-exchange capacity determinations, with results as given below

Material	Millicquivalent
Oat-hull lignin.....	0 062
Do.....	050
Oat-hull lignin, second fraction.....	039
Do.....	038
Corn-cob lignin.....	040
Do.....	036
Flax-shive lignin	
Wet.....	192
Dry.....	151
Flax-shive ligno-hemicellulose	
Wet.....	186
Dry <sup>4</sup> .....	167
Sphagnum-moss lignin	
Wet.....	098
Dry.....	041
Sphagnum-moss ligno-hemicellulose, wet <sup>5</sup> .....	158

According to Phillips, lignin has a molecular weight of about 700 and apparently four hydroxyl groups to which a base might attach. Obviously, only a small fraction of the total theoretical base capacity at optimum reaction is manifested under the conditions of these tests

<sup>4</sup> Contains 32.24 per cent hemicellulose

<sup>5</sup> Contains 18.93 per cent hemicellulose

From these determinations it appears that drying lowers the base-exchange activity of these substances. This may be due to diminution of ingress of soluble salts to the interior of particles. The tendency of these ligneous and lignocellulose fractions to include impurities and to hydrolyze under treatment of base-exchange tests is a cause of some difficulty and uncertainty.

### GENERAL DISCUSSION

The data presented clearly indicate that a portion of the peat profile samples studied is of colloidal size. The particles retained in the centrifuge bowl show some colloidal properties, and it seems probable that the effective size for marked colloidal size is larger for organic than for inorganic colloid. The tendency shown for additions of calcium carbonate to increase base adsorption suggests that such treatment may conserve or produce base-adsorptive complexes. Since the ligneous fraction in these experiments and those of Tenney and Waksman (25, 26) tends to become concentrated as decomposition of organic matter advances, and organic material, such as saw-grass peat or flax shives, of large ligneous content is slow to decay, it appears that the base-exchange capacity of organic residues is fairly permanent. The ligneous character and high base-exchange capacity of old sedimentary layers would appear to support this view.

Chopping or grinding, and control of temperature, moisture, aeration, reaction, and nutrients required by decomposition microorganisms, afford means of regulating the rate of decomposition (28). A knowledge of composition and base-exchange capacity of farm waste or available litter should be helpful. The flash decomposition of legume residues like sweetclover, noted by Smith and Humfeld (23), although they release nutrients and energy, may be wasteful under some circumstances.

When small amounts of peat or ligneous colloid are mixed with sand, the base-exchange activity of such organic colloids may increase with dilution until not more than a monomolecular layer of colloid coating surrounds each sand grain.

Mixing organic and inorganic colloid and supplying calcium carbonate may provide conditions favorable for a high base-exchange capacity of the whole system. A concentration of ions at the interface of a mixture may operate to increase base-exchange capacity. A large proportion of organic matter and the presence of bases sufficient to give a faintly alkaline reaction is indicated as desirable by these studies and is in line with results of Mattson (13). It seems possible that any acidic organic compound or other derivatives containing hydroxyl groups may react with bases, and that ketones or aldehydes may be oxidized to acids which would neutralize bases and perhaps allow base adsorption. Proteins or other amphoteric substances in soil organic matter may also conceivably affect base-exchange capacity of organic soil colloids. The organically combined and also the extraneous inorganic matter may affect the base-exchange capacity of the peaty colloids. Studies of natural or synthetic organic colloids seem a promising field for investigation. It would appear improbable that the base-exchange properties of natural organic soil colloid can be assigned to any definite chemical compound, since the material is the result of the reaction of widely different materials under very

diverse conditions. Comparative results under specified conditions are obtainable

### SUMMARY

Experiments with 8 peat profiles, including 26 soil-layer samples, show that it is possible to separate organic colloids from peat with fairly good, though scarcely quantitative, yields. This natural organic colloid absorbs about 50 per cent more water, over 3.3 per cent sulphuric acid, than the average for inorganic soil colloids. The proximate composition of peat colloids varies widely with parent material and climatic conditions. The colloid fraction is richer in nitrogen than the peat from which it is separated. Although the base-exchange capacity of peat is large, its colloid fraction gives much higher values. The ligneous fraction of the peat colloid, although manifesting comparatively good base-exchange capacity, appears to have this property largely destroyed by treatment with hydrogen peroxide.

Plant materials manifest base-exchange capacity in different degrees, which may affect their comparative values for green manure or stable litter. Studies with composts show a concentration of ligneous material as decomposition proceeds and this is correlated with increased base-exchange capacity. The state of the colloid, or of its ligneous fraction, appears to be conditioned for higher base adsorption by additions of sand, clay, or calcium carbonate. Formation of addition complexes, or the freeing of double-valence bonds, seems probable, and merits further investigation.

Results obtained should be of practical value in composting as well as in economy of soil organic matter and soil fertility.

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# THE DECOMPOSITION OF VETCH GREEN MANURE IN RELATION TO THE SURROUNDING SOIL<sup>1</sup>

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## INTRODUCTION

In two previous papers by Smith and Humfeld (7, 8),<sup>3</sup> studies on the effect of the decomposition of green rye and green vetch, used as green manures on two acid and two neutralized soils, were reported. The results obtained indicated that further studies on the extent of the activities of the microorganisms that caused the decomposition of the organic materials added would be desirable.

In these experiments the green manure turned under was deposited in a layer approximately 5 inches below the surface of the soil. Sampling consisted in taking a number of cores of soil to a depth of 6 inches and then homogenizing the soil from these cores in order to obtain a representative sample. In this operation the green manure was thoroughly mixed with the soil, and if any difference in activity existed in the soil at various depths or in the green manure, these differences tended to be obscured or masked. It was suggested that if any information were to be gained as to the nature or possible localization of the activity of the microorganisms in the layer of green manure and in the soil at different depths, a special method of sampling would have to be developed.

Preliminary observations<sup>4</sup> indicated that there were great differences in the number of organisms in the soil and in the green manures. In order to obtain conclusive data on the relationships of the decomposing green manure to the surrounding soil the following experiment was planned.

## SOIL AND MANURE

Two plots of Leonardtown clay loam were used, one neutralized with ground limestone and the other left in the naturally acid condition. These plots formed part of the series used for the previously reported experiments on green manures (7, 8), but had had no treatment, being kept fallow and at optimum moisture with distilled water. The size of each plot was 1½ feet by 3 feet, and the soil was approximately 10 inches deep. The vetch used as green manure was grown on a separate bench in the greenhouse until approximately a maximum growth was obtained. This corresponds to the stage just before flowering. The vetch was cut close to the soil and 2,500 grams of

<sup>1</sup> Received for publication July 20, 1931, issued March, 1932.

<sup>2</sup> The method of sampling used in this work is a result of a suggestion by Charles Thom, principal mycologist, that a study of the decomposing green manure be made apart from the soil. The authors are indebted to him for this suggestion and for his interest and criticism of the work. They wish to express their appreciation to Daniel Ready, assistant scientific aid, for making the ammonia, nitrate nitrogen, and moisture determinations, and to George Irving, jr., under scientific helper, for making the pH determinations.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 120.

<sup>4</sup> Leaves and stems of rye and vetch were sieved out of subsamples of the green manure studied by Smith and Humfeld. These were examined microscopically by Charles Thom. The enormous number of bacteria found in and on these green materials made necessary a restudy of the whole situation on a different method of sampling, hence this paper.

the green vetch tops were brought to the experimental plots and turned under 5 inches below the surface of the soil. The vetch contained 13.8 per cent dry matter and 3.97 per cent total nitrogen on the dry basis. This is equivalent to about 53,500 pounds of green vetch per acre, or about 7,400 pounds of dry material and 270 pounds of nitrogen per acre.

The plots were sampled immediately after the vetch was turned under, subsequent samples being taken 2, 4, 7, 14, 21, 35, and 56 days after green manuring.

#### METHOD OF SAMPLING

The cores of soil were taken in the usual manner, but instead of mixing the sample obtained, each core was divided carefully into three equal parts: (1) The 0 to 2 inch layer, (2) the 2 to 4 inch layer, and (3) the 4 to 6 inch layer. This last layer contained the green manure, which was separated as thoroughly as possible from the soil and treated as a separate sample. In order to get material enough for analysis twenty 6-inch cores were taken from each plot and fractionated as described. The homologous fractions were combined, homogenized, and samples weighed out for the determination of the number of soil microorganisms and protozoa, amounts of ammonia nitrogen, nitrate nitrogen and moisture, the pH value of the soil and decomposing green manure, and the carbon dioxide evolution from the surface of the soil.

#### METHODS OF ANALYSIS

The number of microorganisms was estimated by plating the appropriate dilutions on soil-extract agar, as described previously (7). The number of protozoa was determined by the dilution method, using dilutions of 1 to 50, 1 to 500, 1 to 5,000, and, when necessary, 1 to 50,000. Tubes of broth<sup>5</sup> were inoculated with a milliliter of these dilutions and incubated 7 to 10 days at 28° C. The presence of protozoa was determined by microscopic examination. Five tubes were inoculated from each dilution. The number of protozoa in the original material was estimated from the number of tubes of the highest dilution showing a growth of protozoa. The counts are reported as number per gram of oven-dry material.

Ammonia nitrogen was determined by distillation with MgO according to the official methods (1), with the following modification. The  $\text{NH}_3$  was collected in 5 per cent  $\text{H}_3\text{BO}_3$  and was titrated with standard 0.14N  $\text{H}_2\text{SO}_4$ , brom phenol blue being used as an indicator, as described by Scales and Harrison (6) and Markley and Hann (3).

Nitric nitrogen in the soil samples was determined by the phenol disulphonic acid method. In the green manure it was determined by treating the residue from the ammonia analysis according to the Zn-Cu couple reduction method (5), boric acid being used as the absorbing agent and the titration being made as described for ammonia nitrogen.

The reaction of the samples was determined by means of the quinhydrone electrode in the usual manner. Moisture determinations were made by drying the samples at 105° C overnight.

<sup>5</sup> Composition of the broth was as follows: Soil extract 1,000 cubic centimeters, and  $\text{K}_2\text{HPO}_4$  0.5 gram. To each test tube containing about 8 milliliters of this broth, there was added a piece of timothy hay long enough to extend well above the surface of the liquid. Sterilization was by autoclaving.

Carbon dioxide evolution was determined as described by Humfeld (2). The method consists of passing a known volume of air over a known and inclosed area of soil and collecting it by means of absorption in potassium hydroxide solution of known strength. The amount of carbon, as carbon dioxide evolved from the soil, was calculated as grams per square meter in 24 hours.

### RESULTS

The results of the plate counts of microorganisms in the soil and in the decomposing green manure are summarized in Table 1.

TABLE 1—*Millions of microorganisms in soil at various depths when unlimed and limed and in fresh and in decomposing green manure*  
[Calculated on the basis of dry weight]

Days after green manuring	Microorganisms in unlimed soil at indicated depth (inches)			Green-manure layer	Days after green manuring	Microorganisms in limed soil at indicated depth (inches)			Green-manure layer
	0 to 2	2 to 4	4 to 6			0 to 2	2 to 4	4 to 6	
	<i>Millions</i>	<i>Millions</i>	<i>Millions</i>	<i>Millions</i>		<i>Millions</i>	<i>Millions</i>	<i>Millions</i>	<i>Millions</i>
0	7 5	7 9	11 1	970	0	43 9	45 6	35 1	970
2	6 4	5 3	7 7	7,350	2	47 6	36 3	20 0	9,800
4	13 4	10 1	11 0	21,500	4	52 6	29 9	31 2	46,400
7	21 0	19 8	20 5	5,430	7	61 3	48 1	66 6	5,000
14	12 5	8 4	13 3	5,800	14	32 0	34 5	23 8	760
21	23 1	17 2	27 5	508	21	60 1	37 0	39 4	420
35	10 6	8 7	8 7	352	35	44 8	47 6	24 0	173
56	13 7	3 7	10 8	73 5	56	75 0	39 6	32 2	87 6

It will be noted that the number of microorganisms in the limed soil was considerably higher than the number in the unlimed soil, and that the fluctuations in number throughout the duration of the experiment were comparatively small. There was a tendency for the number in the different soil layers to increase, the highest counts being obtained in 7 and 21 days. However, when these counts are compared with the counts obtained in the green manure itself, they become insignificant, for while the number of microorganisms in the soil runs into the millions, the number in the green manure runs into the billions. The count of the vetch immediately after it was turned under was 970,000,000 per gram of dry material. This number is much greater than is ordinarily found on growing plants. It probably was due to the fact that the vetch had been grown in the greenhouse and had become closely matted on the soil. As a result, many of the lower leaves were dead. Under the optimum conditions for the growth of microorganisms in the greenhouse, these dead leaves were already undergoing decomposition and contained great numbers of bacteria and protozoa.

After four days the initial count had increased to more than 21,000,000,000 in the decomposing vetch in the acid soil and exceeded 46,000,000,000 in the limed soil. A rapid reduction in numbers took place after this, and 56 days after treatment only 73,000,000 and 88,000,000 were found in the acid and limed soil, respectively. The disappearance of the majority of the organisms in the green manure coincided with the disappearance of the leafy part of the vetch. It is apparent that when the more readily decomposable parts of the vetch had been consumed, the numbers rapidly decreased, as the more resistant stems did not contain sufficient readily available materials to support the great number of microorganisms present.

It is interesting to note the results obtained in counting the protozoa as shown in Table 2.

TABLE 2—Numbers of protozoa in soil at various depths when unlimed and limed and in fresh and decomposing green manure

[Calculated on the basis of drv weight]

Days after green manuring	Protozoa in unlimed soil at indicated depth (inches)			Green-manure layer	Days after green manuring	Protozoa in limed soil at indicated depth (inches)			Green-manure layer
	0 to 2	2 to 4	4 to 6			0 to 2	2 to 4	4 to 6	
0	0	90	60	7,300	0	90	30	30	14,600
2	40	40	0	23,000	2	40	0	80	24,500
4	40	80	40	152,000	4	40	80	0	81,500
7	80	80	0	960	7	40	0	0	890
14	120	30	90	79	14	60	60	0	790
21	60	60	30	750	21	30	60	30	750
35	90	0	0	380	35	0	90	0	1,100
56	90	0	60	75	56	0	0	0	75

The number of protozoa in the different soil layers was small, the variation recorded being attributable to the method of counting. The vetch layer just after it was turned under contained a larger number, but the interesting fact was the increase in numbers of protozoa in the decomposing green manure. These numbers increased rapidly, the peak being reached in four days. After that the count dropped suddenly and remained low.

The number of fungi and actinomycetes was not determined, as all previous studies had shown that there is no significant change in the number of either under these conditions.

TABLE 3—Parts per million of ammonia nitrogen in unlimed and limed soil at various depths, and in fresh and in decomposing green manure

[Calculated on the basis of dry weight]

Days after green manuring	Ammonia nitrogen in unlimed soil at indicated depth (inches)			Green-manure layer	Days after green manuring	Ammonia nitrogen in limed soil at indicated depth (inches)			Green-manure layer
	0 to 2	2 to 4	4 to 6			0 to 2	2 to 4	4 to 6	
0					0				
2			13	620	2	11	8	8	568
4	7	3	8	752	4	1	2	6	1,060
7	10	13	13	142	7	6	9	17	164
14	13	19	56	285	14	8	6	53	174
21	14	23	62	210	21	17	14	25	59
35	11	21	35	65	35	12	12	12	20
56	13	24	26	23	56	6			6

The results obtained from the analyses for ammonia nitrogen are given in Table 3 and show that the amount of this form of nitrogen in the soil was low. However, the amount in the green manure was considerable and was especially high in the first four days of decomposition. As decomposition progressed, the amount of ammonia nitrogen decreased, and at the end of the experiment the amount was no higher than in the surrounding soil. The ammonia nitrogen in the 4 to 6 inch layer, or in the soil adjacent to the green manure, increased to a maximum of 62 parts per million in 21 days in the unlimed soil and to 53 parts per million in 14 days in the limed soil. This was no doubt due to the diffusion of ammonia from the decomposing material.

TABLE 4—Parts per million of nitric nitrogen in unlimed and limed soil at various depths, and in fresh and in decomposing green manure

[Calculated on the basis of dry weight]

Days after green manuring	Nitric nitrogen in unlimed soil at indicated depth (inches)			Green-manure layer	Days after green manuring	Nitric nitrogen in limed soil at indicated depth (inches)			Green-manure layer
	0 to 2	2 to 4	4 to 6			0 to 2	2 to 4	4 to 6	
0	33	39	34	-----	0	59	65	49	-----
2	42	37	35	608	2	35	70	31	570
4	43	39	40	429	4	70	53	41	477
7	62	35	38	30	7	109	51	46	25
14	88	36	36	44	14	144	49	95	59
21	82	42	51	50	21	139	73	88	36
35	116	47	54	74	35	182	67	83	86
56	198	64	60	67	56	439	93	71	74

The nitric nitrogen, as shown in Table 4, in the unlimed soil at the beginning of the experiment was 33 to 39 parts per million, the limed soil contained 49 to 65 parts per million.

In the green manure the nitric nitrogen decreased rapidly, and after seven days only 30 and 25 parts per million were present in the green-manure layer of the acid and limed soils. The outstanding fact is the gradual accumulation of nitric nitrogen of the upper 2-inch layer. In the unlimed soil the increase was from 33 to 198 parts per million, and in the limed soil, from 59 to 439 parts per million.

TABLE 5—pH values of the unlimed and limed soil at various depths and of the fresh and decomposing green manures

Days after green manuring	pH value of unlimed soil at indicated depth (inches)			Green-manure layer	Days after green manuring	pH value of limed soil at indicated depth (inches)			Green-manure layer
	0 to 2	2 to 4	4 to 6			0 to 2	2 to 4	4 to 6	
0	4.3	4.3	4.3	6.8	0	7.3	7.4	7.0	6.7
2	4.2	4.3	4.3	5.5	2	7.3	7.5	6.8	7.3
4	4.2	4.3	4.2	7.3	4	7.5	7.6	6.9	8.1
7	4.1	4.3	4.3	4.7	7	7.2	7.2	6.5	7.6
14	4.2	4.4	4.8	6.6	14	7.2	7.4	7.1	7.7
21	4.1	4.2	4.4	5.5	21	7.2	7.4	6.7	6.6
35	4.0	4.3	4.3	4.6	35	7.3	7.6	7.3	7.3
56	3.9	4.1	4.2	4.3	56	7.0	7.4	7.2	6.8

Table 5 shows the results of the pH determinations. The pH value of the green manure added to the unlimed soil was 6.8, whereas the pH value of the soil was 4.3. However, as the vetch disappeared, the pH value of the green manure decreased, and at the last sampling it was the same as that of the surrounding soil. The surface layer in the meantime had become more acid, perhaps due to the concentration of nitrates there.

In the limed soil the pH value of the green manure was somewhat higher than that of the surrounding soil during the stage of most active decomposition and during the greatest accumulation of ammonia. The difference was not so striking as in the case of the unlimed soil.

The rate of carbon dioxide production is given in Figure 1. The effect of turning under green manure on the evolution of carbon

dioxide was immediate and was marked. The peak of the evolution was reached in 3 days, after which a rapid reduction took place. In 20 days this decline had practically stopped, and a fairly constant rate, which was close to the rate prevailing before the green manure was turned under, was maintained. Apparently the quantity of material turned under was the determining factor. The amount of carbon dioxide given off from both limed and unlimed soil before treatment was negligible when compared to that given off after the addition of the green manure.

### DISCUSSION

In order to gain a clear picture of the effect of the addition of green vetch to this soil, it is necessary to compare the results of each determination with those of every other determination. For instance, if plate counts of microorganisms are compared with the other

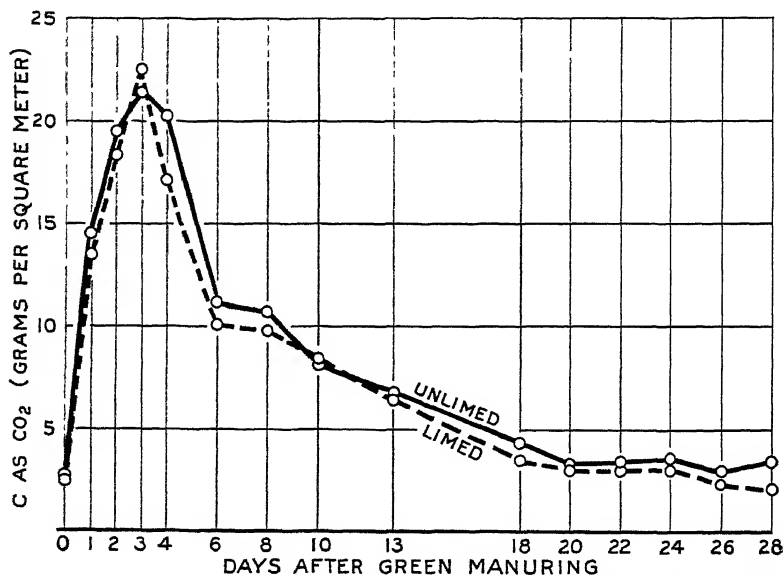


FIGURE 1 —Grams C as CO<sub>2</sub> evolved per square meter of limed and unlimed soil treated with vetch green manure

factors certain correlations are seen. When the plate counts of microorganisms in the green-manure layer were high, as during the first few days, the count of protozoa, the amount of ammonia nitrogen, and the evolution of carbon dioxide were also high, as might be expected. Consequently, at the same time the pH value of the green manure was at the maximum and the nitric-nitrogen content, although still high, was being rapidly reduced.

The decomposition of the green manure causing the great increase in number of microorganisms, which by observation of the plates were found to be almost exclusively bacteria, had practically no effect on the number of microorganisms in the soil itself. Since the green manure contained a great number of microorganisms before it was turned under, it may be said that its decomposition proceeded

independently of the microorganisms in the soil. The same observation may be made as to the number of protozoa. Whether the plant material itself served as a source of energy for the protozoa or whether, as has been suggested by Russell (4), the protozoa subsist on bacteria, can not be stated. However, it was definitely determined that as the number of bacteria increased the number of protozoa increased and also as the number of bacteria decreased the number of protozoa decreased.

The increase in ammonia-nitrogen content of the green manure was no doubt due to the breaking down of the more complex nitrogen compounds in the plant material. Isolation of a number of the bacteria from the colonies obtained from the plate counts of this material showed that most of them were able to ammonify peptone broth readily. This ammonification of the proteins in the plant materials caused an increase in the pH of the material and was, of course, at a maximum when microbial activity was greatest.

The nitric-nitrogen determinations, however, give a somewhat different picture. In the green manure the nitric nitrogen was highest at the beginning and decreased rapidly up to seven days after the vetch was turned under. After that it remained approximately constant. It is evident that nitrification of the ammonia was constantly taking place, because, although the amount of ammonia in the soil adjacent to the green manure was always somewhat higher after the first seven days than in the soil more distant from the green-manure layer, an accumulation of ammonia did not take place, but a gradual accumulation of nitric nitrogen occurred in the upper 2 inches of soil. This accumulation took place both in the naturally acid and in the limed soil. It is very evident that although the soil of the unlimed plot reached a very low pH value (3.9), nitrification was sufficiently rapid to insure the oxidation of the ammonia given off by the green manure.

It is not clear from the data at hand whether the increase in nitric nitrogen was due to nitrification at or near the surface of the soil, or whether the accumulation was the result of nitrification at greater depths and the transportation of the nitrates to the surface by the physical action of capillarity and surface evaporation. Some observations as to the concentration of nitrates at the surface under the conditions obtaining during this and similar experiments have shown that a great proportion of the total nitrates in the soil may be concentrated at the surface. Concentrations of 1,300 parts per million have been found in the upper one-quarter inch of soil. It has been assumed that the nitrates were carried to the surface by capillary action, where they were left by the evaporation of the water. Periodic sprinkling should dissolve these nitrates and distribute them through the soil, but apparently it did not do so.

As the number of microorganisms in the green manure decreased, the nitric-nitrogen accumulation in the upper 2 inches of soil increased. This was in agreement with previous findings (7, 8).

The carbon-dioxide evolution correlated very nicely with the other activities, the peak of carbon-dioxide evolution coinciding with the time at which the greatest number of microorganisms were found in the green manure. It may be said that under the conditions of the experiment the carbon-dioxide evolution was a good indication of the microbial activity in the material undergoing decomposition.

## SUMMARY

An experiment on the decomposition of green vetch added to a naturally acid and a limed soil is reported

A special method of sampling was used in which the 6-inch cores of soil were divided in three successive 2-inch layers. The third layer contained the layer of green manure, which was separated from the soil as thoroughly as possible and treated as a separate sample.

Twenty fractionated cores from each plot were homogenized, and the representative samples of each layer obtained were analyzed for number of microorganisms as indicated by plate count on soil-extract agar, number of protozoa, and amount of ammonia nitrogen, nitric nitrogen, and moisture. The soil reaction (pH) and carbon-dioxide evolution were also determined.

The results show that the increase in number of bacteria and protozoa was limited almost entirely to the green-manure layer. The soil acted as a blanket, insuring more or less uniform conditions of temperature and moisture, and as an absorbent for the ammonia produced in the decomposition of the green manure. In all probability the soil was the material in which nitrification of this ammonia took place. It was also found that rapid nitrification took place even in the very acid soil and that an accumulation of nitrates occurred at the surface.

The pH of the green manure was shown to be higher than the pH of the surrounding soil during the period of active decomposition. Carbon-dioxide production was correlated with microbial activity and was considered to be a good indicator of such activity under the conditions of the experiment.

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# INHERITANCE OF RESISTANCE TO BUNT, *TILLETIA TRITICI*, IN CROSSES OF WHITE FEDERATION WITH TURKEY WHEATS<sup>1</sup>

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## INTRODUCTION

In the study of the inheritance of resistance to bunt, *Tilletia tritici* (Bjerk.) Wint., it is desirable to know the number of resistant factors present in each resistant variety and the effect of each factor. Also it is necessary to know whether or not various resistant varieties contain the same or different factors for resistance if such varieties are to be used intelligently for breeding other resistant varieties. Crosses are made with appropriate test varieties, as soon as they are available, in order to determine this last point. Martin wheat (*1*)<sup>3</sup> has a single dominant factor for resistance to bunt and is used as a test variety for this factor, which is designated as the Martin factor. The only other test variety now available is selection 1418. This selection contains the second Hussar factor (*3*) which allows bunt to develop on about half the heterozygous plants. However, this selection was not available when the investigations with Turkey C. I.<sup>4</sup> 1558 and Turkey C. I. 3055 were begun.

This paper deals with crosses of susceptible White Federation with resistant Turkey C. I. 1558 and Turkey C. I. 3055 wheats from which the number of factors for resistance and their effect may be determined. These two Turkey wheats also were crossed with Martin to see whether the Martin factor was present. Recently appropriate crosses were made to determine whether or not the factors present in these two Turkey wheats were identical with each other and the same as the second Hussar factor. These data will not be available for two or three years.

The literature relating to inheritance of resistance to bunt in wheat has been reviewed and discussed in previous publications (*1, 3, 4*).

## METHODS AND MATERIALS

The parental material and hybrid populations were grown in the field at University Farm, Davis, Calif. Conditions there favor such investigations because relatively high bunt infection can be obtained when wheat is sown in the fall. Both spring and winter varieties may be seeded at that time without any danger of winter killing and with the assurance that both types will mature the following summer.

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<sup>2</sup> The writer acknowledges valuable suggestions from R. E. Clausen, Division of Genetics, and various members of the Division of Agronomy, University of California, and from various members of the Division of Cereal Crops and Diseases.

<sup>3</sup> Reference is made by number (*italic*) to Literature Cited, p. 126.

<sup>4</sup> C. I. refers to accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

The seeds were thoroughly blackened with bunt spores. The inoculum was collected by W. W. Mackie in 1917 on Little Club wheat in the Montezuma Hills district of Solano County, Calif. It was propagated by Mackie on Little Club wheat in the botany garden at Berkeley, Calif. Since 1919 the writer has grown bunt from this original collection on White Federation wheat at Davis. The inoculum used, therefore, has been derived from one original collection of bunt. Since Faris (5) has shown that physiologic forms of bunt exist, the writer has been careful not to introduce new collections of bunt into the nursery. The fact that the same collection of bunt has been used continuously at Davis makes it reasonably certain that the same physiologic form, or possibly a mixture of forms, has been employed in all these bunt investigations. This is indicated also by the fairly constant way in which the parental wheat varieties have reacted to this inoculum. This collection of bunt has been designated by Reed (6) as Physiologic Race III of *Tilletia tritici*.

The wheat seeds were spaced from 2 to 3 inches apart in rod rows 1 foot apart. The entire nursery was sown within three or four days in order to avoid the effects of different temperatures and soil moistures. At harvest time the plants in each row were pulled and separated into two piles, bunt free and bunted. The total number of plants and the number of bunted plants were recorded, and the percentage of bunt infection was calculated. A plant was classified as bunted if it showed any infection.

The percentages of bunt produced by the parent varieties may be seen in Table 1.

TABLE 1.—Annual percentage of bunt infection in the parent wheat varieties from 1920 to 1922 and 1927 to 1929, when grown at Davis, Calif.

Variety	Percentage of bunted plants						
	1920	1921	1922	1927	1928	1929	Average
Turkey C I 1558.....	14.5	0.3	0.5	0	2.8	0	3.00
Turkey C I 3055.....	0	0	0	0	2	1	0.05
Martin.....	0	0	0	0	0	0	0
White Federation.....	88.7	51.6	58.3	66.6	68.9	78.6	68.80

Turkey C I 1558 had 14.5 per cent of bunted plants in 1920. Since that time the percentage of bunt in this variety has been much lower, reaching a maximum of 2.8 per cent in 1928. The reasons for the comparatively high percentage of diseased plants in 1920 are not apparent. Tisdale et al. (7) report an average of almost 9 per cent of bunted heads for this variety at Moro, Oreg., in 1919 and 1920.

Turkey C. I. 3055 has been almost free from bunt, producing only a little in 1928 and 1929. This variety produced 1 per cent of bunt in 1919 but was bunt free in 1920 at Moro, Oreg. (7). Martin has been entirely free from bunt at Davis, while White Federation, the susceptible parent, has produced more than 50 per cent of diseased plants each year.

#### EXPERIMENTAL RESULTS

All the crosses were made in 1926. The  $F_1$  seeds were not inoculated because of the small number available.

A part of the  $F_2$  seeds of all crosses was treated with copper carbonate to protect them from bunt infection so that a supply might

be grown for  $F_3$ . Enough seeds to plant approximately 20 rod rows of each cross were inoculated and grown in 1928.

The  $F_2$  data do not permit a satisfactory genetic analysis because some susceptible plants usually escape infection. Some resistant plants occasionally also become partly infected. The data do give some idea of the number of factors present and indicate the percentage of bunted plants that may be expected in  $F_3$  rows of the same genotype. The data collected in  $F_2$  are recorded in Table 2.

TABLE 2—*Percentage of bunted plants in parents and  $F_2$  of the crosses named when grown in the field at University Farm, Davis, Calif., 1929*

Parent or cross	Total plants	Bunted plants	
	Number	Number	Per cent
Turkey C I 1558.....	504	14	2.8
Turkey C I 3055.....	479	1	.2
White Federation.....	309	253	81.9
White Federation $\times$ Turkey C I 1558.....	815	421	51.6
White Federation $\times$ Turkey C I 3055.....	623	252	40.4
Martin $\times$ Turkey C I 1558.....	921	43	4.7
Martin $\times$ Turkey C I 3055.....	1,016	50	4.9

There was 51.6 per cent of bunted plants in the  $F_2$  of White Federation  $\times$  Turkey C I 1558 as compared with 40.4 per cent in the cross with Turkey C I 3055. The selection 1418, which carries the second Hussar factor, produced 53.3 per cent of bunted plants in  $F_2$  when crossed with Little Club (3). The  $F_2$  data, then, indicate that both strains of Turkey contain single factors for resistance to bunt which are similar in effect to the second Hussar factor.

The  $F_2$  of Martin  $\times$  Turkey C I. 1558 and Martin  $\times$  Turkey C. I. 3055 contained 4.7 and 4.9 per cent of bunted plants, respectively, showing that the Martin factor is not present in these varieties.

In the  $F_3$ , 299 rod rows were grown from 299  $F_2$  plants of the cross White Federation  $\times$  Turkey C I 1558. There were grown also 296  $F_3$  rows of White Federation  $\times$  Turkey C I 3055, 183 rows of Martin  $\times$  Turkey C I 1558, and 190 rows of Martin  $\times$  Turkey C I 3055. The  $F_2$  plants from which these  $F_3$  rows were grown had been protected from bunt by seed treatment. There were from 30 to 60 plants in each  $F_3$  row. The classification of  $F_2$  plants on the basis of the behavior of their progeny in  $F_3$  rows is more reliable than classification in  $F_2$ . The  $F_3$  data are shown in Table 3.

The number of rows in the 0 to 5 per cent class were separated into those with no bunted plants and those with 1 to 5 per cent of bunted plants because of the special interest in the former. The nature of the distribution of White Federation  $\times$  Turkey may be seen more readily from Figure 1. In each cross the number of rows under the three modes agrees satisfactorily with the 1:2:1 ratio. Accepting the minima as they occur, the number of rows for White Federation  $\times$  Turkey C. I. 1558 was 79.0:154.5:76.5 where the number expected was 77.5:155:77.5. In the cross with Turkey C. I. 3055 there were 64.5:155.0:76.5 where 74:148:74 were expected. The minima perhaps should not be thought of as representing with absolute accuracy the divisions between phenotypes. The agreement between the two crosses is good, and the minima fall practically at

the same points as those obtained in the cross with the second Hussar factor (3). In the latter case the first minimum occurred at 17.5 and the second one at 67.5 per cent of bunt infection.

TABLE 3—Distribution of parent and  $F_2$  rows of the crosses named into 5 per cent classes for bunt infection, when grown at Davis, Calif., 1929

Parent or cross	Distribution of rows by percentage classes for bunt infection										
	0	1-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50
Turkey C I 1558.....	12										
Turkey C I 3055.....	11	1									
White Federation.....											
Martin.....	6										
White Federation $\times$ Turkey C I 1558.....	27	34	10	6	4	6	9	24	31	27	21
White Federation $\times$ Turkey C I 3055.....	36	19	8	3	6	17	25	28	24	19	15
Martin $\times$ Turkey C I 1558.....	73	24	29	16	12	11	6	1	3	2	2
Martin $\times$ Turkey C I 3055.....	89	38	17	16	12	4	3		2		2

Parent or cross	Distribution of rows by percentage classes for bunt infection										Total number rows
	51-55	56-60	61-65	66-70	71-75	76-80	81-85	86-90	91-95	96-100	
Turkey C I 1558.....											12
Turkey C I 3055.....											12
White Federation.....	1	1				1	8	7	4	1	23
Martin.....											6
White Federation $\times$ Turkey C I 1558.....	17	10	6	3	6	8	9	23	13	5	299
White Federation $\times$ Turkey C I 3055.....	10	6	2	3	2	6	17	26	16	8	296
Martin $\times$ Turkey C I 1558.....	1	1	1								183
Martin $\times$ Turkey C I 3055.....		1	1		2		1		1	1	190

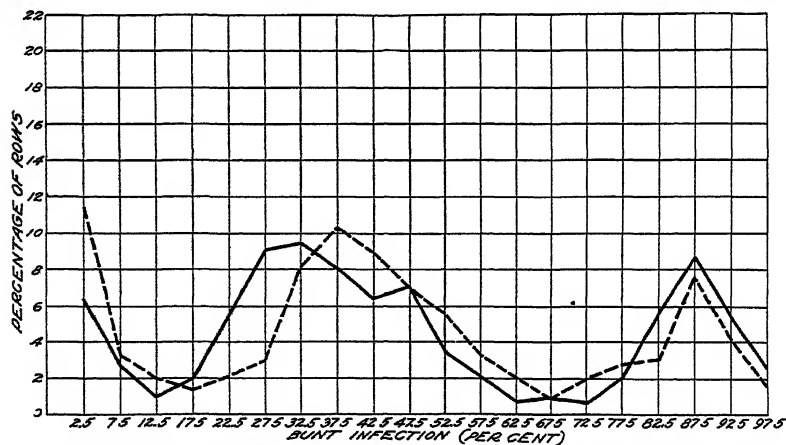


FIGURE 1—Distribution of  $F_2$  plants on the basis of  $F_3$  rows of the crosses White Federation  $\times$  Turkey C. I. 1558 wheat (broken line) and White Federation  $\times$  Turkey C. I. 3055 wheat (solid line) into 5 per cent classes for bunt infection

Although the agreement between the two crosses is good, it is obvious that there is a higher percentage of bunt in the heterozygous  $F_2$  rows of the Turkey C. I. 1558 cross than in the Turkey C. I. 3055 cross. The former had an average of 41.5 per cent of bunted plants and the latter 36.3. It will be recalled that the  $F_2$  of White Federa-

tion  $\times$  Turkey C. I. 1558 produced 51.6 per cent of diseased plants, as compared with 40.4 in the Turkey C. I. 3055 cross. The reason for the more frequent occurrence of bunt on heterozygous plants is not known definitely but it may be due in part to modifying factors. In Table 1 Turkey C. I. 1558 was shown to be a little more susceptible to bunt than Turkey C. I. 3055. That small differences in the amount of bunt in resistant wheats may be due to the presence of modifying factors has been shown in an earlier publication (2). If the higher percentage of bunt in heterozygous rows of White Federation  $\times$  Turkey C. I. 1558 is due to the presence of modifying factors, the percentage of bunt in resistant and susceptible rows likewise should be higher. The resistant rows of the Turkey C. I. 1558 cross have an average of 3.1 per cent of bunt as compared with 1.9 per cent for the Turkey C. I. 3055 cross, but the reverse is true for the susceptible rows. The former cross had 84 per cent of diseased plants as against 86.9 per cent for the Turkey C. I. 3055 cross. The differences are not great and do not change the general conclusions that Turkey C. I. 1558 and Turkey C. I. 3055 differ from White Federation in one main factor for resistance. These factors are similar to each other in effect and also are similar in effect to the second Hussar factor. Investigations are under way to determine whether these factors are identical with each other and are the same as the second Hussar factor.

The data obtained from the crosses of White Federation with Turkey C. I. 1558 and Turkey C. I. 3055 demonstrate clearly that the factors for resistance to bunt in these two strains of Turkey are different from the factor in Martin. Because the crosses with Martin were available, they were carried through the  $F_3$ . The presence of bunt in  $F_2$  of Martin  $\times$  Turkey C. I. 1558 and Martin  $\times$  Turkey C. I. 3055 and the presence of susceptible and segregating rows in  $F_3$  of these crosses (Table 3) confirm the conclusion that the Martin factor is not present in either of these strains of Turkey wheat.

#### SUMMARY

The bunt inoculum used in these experiments was derived from one original collection, designated by Reed as Physiologic Race III of *Tilletia tritici*.

Turkey C. I. 1558 and Turkey C. I. 3055, the resistant parents, are very resistant to bunt as compared with White Federation wheat, which produced more than 50 per cent of diseased plants under the conditions of these experiments.

Turkey C. I. 1558 and Turkey C. I. 3055 were crossed with White Federation to determine the number of factors for resistance to bunt in these strains of Turkey and to find out the effect of these factors. They were crossed with Martin to see whether they contained the Martin factor for resistance to bunt.

In the crosses with White Federation the classification of  $F_2$  plants on the basis of the behavior of their progeny in  $F_3$  rows showed that Turkey C. I. 1558 and Turkey C. I. 3055 each differ from White Federation in one main factor for resistance to bunt. These factors are similar in effect to each other and resemble the second Hussar factor in that about half the heterozygous plants become infected. Therefore, they differ from the factor for resistance to bunt in Mar-

tin, which is completely dominant. This point is confirmed further by the presence of bunt in  $F_2$  of crosses of Martin with Turkey C I 1558 and Turkey C I 3055 and by the presence of susceptible and segregating  $F_3$  rows of the same crosses.

Investigations are under way to determine whether or not the factor in Turkey C I 1558 is identical with the factor in Turkey C I 3055 and the same as the second Hussar factor.

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# FLOWERING BEHAVIOR OF THE HOG PEANUT IN RESPONSE TO LENGTH OF DAY<sup>1</sup>

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## INTRODUCTION

One of the most interesting wild plants of the flora of the District of Columbia is the legume known as the hog peanut, *Falcata comosa* (L.) Kuntze. This plant grows best in rich damp soil in woods and thickets. In the writer's garden, developed by reclaiming a magnolia bog, this plant has stubbornly persisted as a most troublesome weed.

In Gray's Manual of Botany<sup>2</sup> it is stated that the hog peanut is a low and slender perennial. This plant as observed by the writer at Washington, D. C., is invariably an annual. This observation agrees with that of Adeline F. Schively,<sup>3</sup> who made a very careful study of its flowering behavior many years ago in Pennsylvania.

## KINDS OF FLOWERS NORMALLY PRODUCED IN THE WILD STATE IN SUMMER

In many respects the flowering behavior of the hog peanut is unique. The production of flowers and seed is especially striking, since it is usual for the plants to produce each season flat aerial pods with small dry seeds, and in addition indehiscent subterranean pods with large fleshy seeds of distinctive character.

In the course of the season the hog peanut normally produces several types of blossoms, ranging on the one hand from strictly open, showy blue or whitish chasmogamic blossoms in small aerial racemes to strictly cleistogamic blossoms, some of which, on the tips of long slender stems, bury themselves in the upper layers of the soil and produce the true hypogean beans of the large fleshy type.

Among the more distinct forms of floral expression in the blossoming series several may be mentioned. The showy colored chasmogamic blossoms of midsummer, which develop as aerial flowers from the uppermost branches of the plant, appear to be the fully developed blossoms of the species. From the higher branches of the plant aerial greenish cleistogamic blossoms are likewise produced. Both types of aerial blossoms produce small, dry, dark-colored aerial pods with one to three small hard seeds. The showy perfect flowers appear only during the midsummer season. Plants grown in the greenhouse in winter never produce the blue showy type of flower, but greenish cleistogamic flowers develop and form small thin orbicular pods.

<sup>1</sup> Received for publication July 14, 1931, issued March, 1932.

<sup>2</sup> [GRAY, A.] GRAY'S NEW MANUAL OF BOTANY. A HANDBOOK OF THE FLOWERING PLANTS AND FERNS OF THE CENTRAL AND NORTHEASTERN UNITED STATES AND ADJACENT CANADA. Rearranged and ext. rev. by B. L. Robinson and M. L. Fernald. Ed. 7, 926 p., illus. New York, Cincinnati [etc.]. 1908. (See p. 530.)

<sup>3</sup> SCHIVELY, A. F. CONTRIBUTIONS TO THE LIFE HISTORY OF AMPHICARPEA MONOICA. Penn. Univ. Contrib. Bot. Lab., 1: 270-363, illus. 1897.

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These are even smaller, more orbicular, and less obviously stalked than the pods produced by the similar greenish cleistogamic summer flowers

In addition to the aerial cleistogamic flowers of summer and winter, long filiform stems bearing at their tips extremely reduced cleistogamic blossoms are formed. These normally push beneath the soil and produce the large fleshy subterranean beans. These beans are produced very abundantly under specific conditions during the entire summer and likewise constitute the common form of floral expression on winter-grown plants.

It would appear that the entire series of flowers merely represents more or less distinct gradations of floral reduction from the normal showy colored blossoms to the merest rudiments found in the most extremely reduced hypogean forms. So complete can this floral reduction become that even the number and size of the stamens and the stigma itself are affected. In the strictly subterranean flowers there is little indication of the normal elongate style, and the capitate stigma seen in the blue flowers is entirely wanting.

The fact that several types of flowers and beans are normally produced in a rather definite sequence throughout the season led Schively to investigate the behavior of the plant for the purpose of determining the factors responsible for these reproductive differentiations. Her observations and work make a very interesting contribution to the unique behavior of this wild bean; but since at the time of her investigations nothing was known of the length-of-day responses of plants, she failed to recognize any specific response that could be attributed to definite quantitative conditions of the seasonal environment.

#### SEASONAL RELATIONSHIPS OF FLOWERING

Consideration may now be given the seasonal relationships of flowering and the normal sequences observed for the production of the several types of flowers from the time the beans first germinate in late April until the plants die in autumn.

In a locality where the hog peanut grows abundantly a heavy crop of viable hypogean seed is usually produced. These are actually self-sown, as the blossom-tipped axillary runners bury themselves wherever possible in the uppermost soil layers and these yield a crop of hypogean beans. Plants may arise from the smaller aerial seed, but the viability and germination of these seeds from aerial blossoms seem far less positive.

Toward the last of April certain areas of the writer's garden are covered with thick stands of young hog-peanut plants arising from the abundant hypogean beans buried in the soil. The cotyledons of the hog peanut, unlike those of the soybean or the common garden beans, remain buried beneath the soil. As the young plants grow in May and June, a tangle of competing vines is produced, and the struggling plants become more or less vigorous climbers when they find at hand supporting weeds and shrubbery.

In mid June or early July, slender, drooping filiform runners sometimes appear, arising even from the shoots that develop from the cotyledonary axils beneath the soil. From the time these first runners with their extremely rudimentary flowers are developed runners are produced in abundance from the aerial leaf axils until

the end of the season, or as long as the plants remain in a vigorous growing condition. There is considerable individual variation, however, in the time of first appearance of these filiform stems with their cleistogamic flowers. On some plants their appearance is delayed as late as the last week of July or early August. This behavior is not anomalous, however, for the normal field assemblage, not having undergone rigorous natural selection for particular degrees of earliness or lateness, would be likely to include individuals of rather wide variability in this respect.

Several weeks after the filiform runners with the hypogean type of blossoms appear, aerial flowers, either greenish and cleistogamic or perfect and bluish colored, usually make their appearance. Schively<sup>4</sup> states that the aerial cleistogamic flowers do not appear until the showy blue aerial flowers are in evidence. This is not an invariable sequence, however, for the writer has observed that the greenish, more reduced cleistogamic aerial flowers have occasionally preceded a little the showy aerial ones, sometimes making their appearance during the first week of August, while the blue flowers did not appear until about August 20. Near the middle of August the blue aerial flowers appear either singly or in racemes.

#### WINTER FLOWERING IN THE GREENHOUSE

Naturally the winter type of aerial cleistogamic blossoms must be confined to artificially grown greenhouse material. However, it would appear that this type of flower is in some respects only a more reduced and more rudimentary floral structure than that produced by the plants in summer, when growth is favored by conditions of better illumination, long days, and perhaps more favorable moisture and temperature conditions. There is reason to believe that the winter type of cleistogamic flower and the resulting legume are but a near approach to the more extreme hypogean type produced upon the filiform runners. This is evidenced by the more extreme reduction in certain features of the style and stigma, and in the smaller, more orbicular pods, that, like the hypogean pods, usually contain but one seed.

#### RESPONSE OF THE PLANTS TO LENGTH OF DAY IN SUMMER

In 1925 and again in 1930 studies were made of the response of the hog peanut to different lengths of day. These studies have afforded a clearer understanding of the normal field behavior of the plant in a wild state. They have also suggested reasons for some of the floral differentiations and apparently established floral sequences throughout the season.

Tiny plants which had germinated from hypogean beans in late April and early May were taken from the wild state and planted in buckets. These were subjected to the following series of day lengths: 5, 8, 10, 12, 13, and 13½ hours and the full length of day of summer at Washington, D. C., i. e., 14 hours and 54 minutes from sunrise to sunset. In one test the plants were darkened from 10 a. m. to 2 p. m. each day.

Filiform runners bearing extremely rudimentary flowers were soon produced on plants under all lengths of day except the controls (full

<sup>4</sup> SCHIVELY, ADELINE F. *Op cit*, p. 325 (See footnote 3)

day, and the plants darkened in the middle of the day. In experiments carried out in 1930 with the different day-length treatments beginning May 2, filiform stems appearing May 29 reached the following lengths on June 2:

	Inches
8 hours daylight.....	5
10 hours daylight.....	4
12 hours daylight.....	3½
13 hours daylight.....	5½
13½ hours daylight.....	1½

Closed aerial flowers appeared on plants growing under the shortened day lengths on the following dates. On the 8-hour, 10-hour, and 12-hour days, June 10; 13-hour day, June 12; 13½-hour day, June 14 (Fig 1)

Showy, bluish, completely developed flowers appeared only on the plants experiencing a day length of 13½ hours and the full length of day. On the plants exposed to a 13½-hour day the flowers appeared on June 23 but on those exposed to full length of day the flowers did not appear until August 12. At no time during the season did the plants darkened in the middle of the day produce the showy bluish type of flower.

Filiform stems began to develop on these plants and on the control plants simultaneously on July 22, followed somewhat later by the closed aerial greenish cleistogamic flowers

Figure 2 affords a comparison of plants grown under different lengths of exposure to daylight

These tests and earlier experiments conducted in 1925 indicate that the length of day exerts an immediate and profound influence upon the type of blossoms produced by the hog peanut

It is evident that an appropriate length of day not only controls the type of floral expression but also completely changes the normal sequences of flowering behavior as usually observed in wild plants in the field. Lengths of day of 13½ hours or less have favored a rapid development of axillary filiform stems bearing the extremely reduced hypogean type of blossoms. A length of day of not less than 13½ hours was required to produce the perfect showy aerial type of blossoms. The green cleistogamic summer type of blossom appears to represent an intermediate form not far removed from the showy flowers, since they are normally produced on the control plants very near the time when the showy blossoms themselves appear. These showy flowers, representing the typical fully developed blossoms of the hog peanut, are obviously favored by the longer midsummer day obtaining in this region.

The extremely rapid growth and excessive production of filiform stems bearing the hypogean type of flower is a remarkable response of the plants to the shorter lengths of day. Day lengths of 13 and 13½ hours were especially favorable to the growth of these runners or stolonlike stems, as shown in Figures 1 and 2. This excessive runner growth was coincident with a more rapid and vigorous growth of the plants themselves in leafiness and in stature. It would appear that the greatest reduction toward a rudimentary condition in the normal flower accompanied the condition of most excessive vegetative growth. As a matter of fact, these filiform runnerlike stems represent extreme attenuations of aerial stem growth bearing the



FIGURE 1—Hog peanut grown under a 10-hour day, beginning April 28. Only greenish aerial flowers and filiform stems bearing hypogean flowers were produced. Closed flowers appeared June 10. Filiform stems appeared somewhat earlier. Aerial pods and small seeds are shown, and likewise the hypogean pods with their single large fleshy seeds, some of them still attached to the underground runners. The slender stems at the right represent runners from aerial leaf axils and show the extreme reduction of the leaves and the minute rudimentary flowers at the tips. (Photographed August 10. About one-half natural size.)

inflorescence, and the leaves are greatly reduced until oftentimes they are represented by mere stipules. Upon these excessively active growing floral stems, which may reach 6 feet or more in length, usually solitary flowers are borne at the tips.

A striking feature of this phase of sexual expression in the hog peanut is the localization of extremely vigorous growth energy in the runners bearing the hypogean type of flowers. It would seem that the axillary branches are suddenly stimulated to become more or less specialized inflorescence branches given over almost entirely to sexual reproduction. It is now well known that when many plants with a distinct vegetative stem, as in the case of the cosmos, are forced into flowering by suddenly reducing the length of day, the reproductive cycle is established by a marked elongation of the terminal inflorescence stem and branches. In these plants, however, with a more



FIGURE 2—Hog peanut grown under different day-length treatments. Tests began with wild plants taken from the field grown from naturally sown hypogean seed May 6, photographed June 18. Left to right: Controls, experiencing the full summer day, 12-hour day, 13-hour day, 13½-hour day, darkened from 10 a. m. to 2 p. m. each day. The greater abundance and length of the filiform stems bearing the hypogean type of flower is well shown on the plants exposed to a 13-hour and a 13½-hour day. At this time the controls showed no flowers of any form whatever.

or less strictly terminal inflorescence, although there may be a reduction of the true foliage leaves to the condition of mere bracts, there is no noticeable reduction of the flowers themselves to a rudimentary condition. The behavior of the hog peanut is probably comparable to this, since a favorable length of day stimulates a similar marked elongation of the inflorescence branches, which arise from nearly all of the older leaf axils. So marked is the response toward reproductive vigor, however, that the weak floral stems develop extremely long internodes and become leafless, and the flowers themselves undergo extreme degeneration to the merest rudiments in comparison with the normal perfect showy blossom.

Coincident with the rapid growth of the axillary filiform stems bearing the reduced cleistogamic flowers, the hypogean pods develop with great rapidity. It is evident that the plants have transferred their energies to these long reproductive stems, with a rapid mobilization of material in all those beans that have a hypogean environ-

ment This very rapid growth of the axillary stems producing the hypogean type of flowers does not exert any unfavorable action upon the fertility of the hypogean beans, for these are usually more viable than the small aerial beans and from the outset produce larger and more vigorous plants This behavior of the hog peanut is similar to that of a number of other plants that regularly produce conspicuous or chasmogamic flowers and cleistogamic flowers In the case of *Polygala polygama* Walt and *P. pauciflora* Willd the chasmogamic flowers are very uncertain in the production of seed, while the cleistogamic flowers produce seed in abundance.

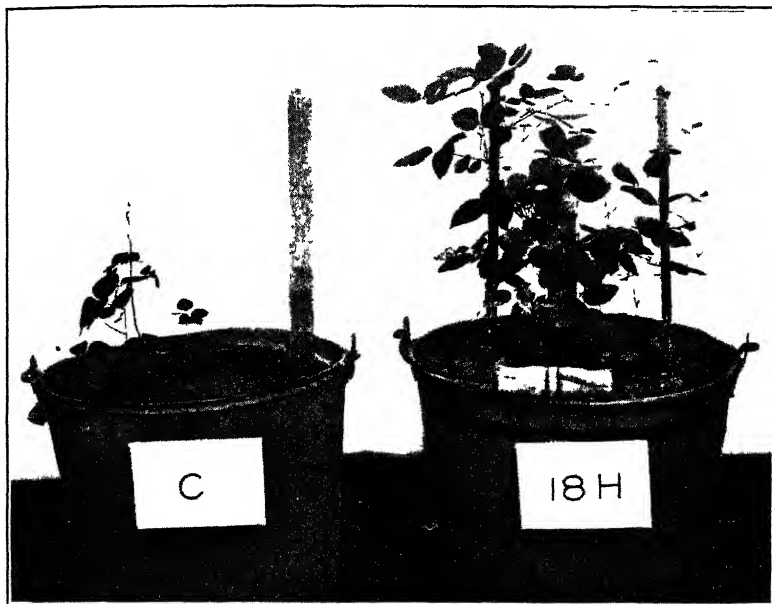


FIGURE 3—Winter-grown plants of the hog peanut obtained from seed of aerial legumes sown October 31, 1930, in the greenhouse, germinated November 30, and photographed January 26, 1931. Plants at right were exposed to light turned on each day from sunset till midnight. The controls at left experienced the normal seasonal length of day and produced the hypogean type of cleistogamic flower. Aerial flowers of the greenish cleistogamic winter type which developed pods were also in evidence January 8.

#### RESPONSE OF THE PLANTS TO ARTIFICIAL LIGHT IN WINTER

Experiments have been carried out during the winter with artificial light (Figs 3 and 4). The plants were grown from the small dry seeds of aerial pods which were sown October 31, 1930, and germinated November 30. Four plants were grown in a bucket. Beginning December 17, a 100-watt light with reflector 1 foot above the plants was kept on from sunset until midnight each day. These plants were stimulated to grow rather vigorously, and became twiners. A few weak filiform stems were in evidence December 19, bearing the rudimentary hypogean type of cleistogamic blossom, but none appeared to produce seed. At no time was there any evidence of aerial flowers, both the greenish winter type of cleistogamic flower and the perfect bluish blossoms being entirely suppressed.

The controls experiencing the normal winter day throughout the experiment developed filiform stems bearing the reduced hypogean type of flower. In addition a number of aerial flowers of the greenish



FIGURE 4.—Plants of the hog peanut of the winter type, grown from aerial seed. The plant at the right was grown with the addition of artificial light each day. Only filiform type of stem bearing the hypogean flowers had developed, all aerial forms of blossoms being suppressed. No pods had developed on these filiform stems when photographed January 26, 1931. At the left is a control plant grown without the addition of artificial light to supplement the winter length of day, with pods developed from aerial cleistogamic flowers. These pods are small, orbicular, 1-seeded, and appear to represent a nearer approach to the hypogean type of pod than the pods developed from aerial cleistogamic or chasmogamic flowers in summer. Filiform stems bearing the typical hypogean type of pod are also shown.

cleistogamic type were produced, from which developed the small orbicular, mostly 1-seeded type of aerial pods characteristic of winter-grown plants.

The weak development of filiform runners on the plants subjected to additional electric light at sunset appears to have resulted from the delay in beginning the experiment. The plants had grown 17 days from germination before the normal length of day had been extended by electric light. It would appear that the additional light had stimulated the plants grown from aerial seed to greater vigor, comparable to that shown at the outset by plants grown from hypogean beans.

In a second test hypogean beans were dug from the soil in the writer's garden and planted in pots January 19, 1931. On January 30, when these had come up, one bucket containing six plants was placed beneath a 100-watt light, which was kept on from sunset till midnight. Axillary filiform stems did not develop, but instead short, thick vegetative stems appeared. The controls produced long, axillary stems, which appeared on February 11 and grew vigorously.

### DISCUSSION

From the data presented it is obvious that, other conditions being uniform, flowering in the hog peanut may be very largely controlled by the length of the day to which the plants are exposed. It would appear that in the field the usual sequences of flowering with respect to the type of blossoms produced are controlled more or less by the normal seasonal changes in the length of day from spring until autumn. Experiments with constant lengths of day indicate that filiform stems bearing rudimentary hypogean flowers are stimulated to appear under day lengths as low as 5 hours, and continue to develop through an increasing series up to at least 13½ hours. At some point between 13½ hours and 15 hours, which is the maximum length of day in the locality of Washington, D. C., it would appear that the days may become too long for an immediate production of this type of inflorescence. This is shown by the fact that control plants did not begin to produce filiform stems until July 22, while plants exposed to a constant day length of 13½ hours began to produce them with great vigor by May 29.

The greenish cleistogamic aerial flowers may somewhat precede the blue aerial flowers, or they may appear after the latter have developed. In some of the experiments these closed aerial flowers appeared August 12, while the blue flowers appeared August 20. In the field the blue aerial flowers usually appear in late July or in August, to be replaced later in the summer by the greenish aerial cleistogamic flowers. The latter apparently represent a response to shorter lengths of day than are favorable to the production of the blue aerial type.

It appears that the hypogean beans are more abundantly produced than the aerial legumes. The former not only germinate with much more certainty but actually produce more vigorous seedlings. These facts would indicate that the perpetuation of the species is more dependent upon the hypogean seed than upon seed developed in aerial legumes. The rapidity of development of the hypogean seeds, which appears to be associated with the extremely rapid growth of the filiform runners bearing them, is an additional accomplishment of value to the species, since it allows an abundance of seed to be produced late in the season at a time when rapid development is most needed.

Since the hypogean beans can be produced under very short lengths of day, it is obvious that the plant need not depend upon the more uncertain aerial beans for reproduction, even in regions where strictly aerial flowers are not able to set seed. It is probably true that the smaller and extremely hard, dry, aerial beans are better adapted to distribution, perhaps by birds or other agencies, than the larger, softer, self-planted terrestrial beans. From the fact that these can develop under very short lengths of day, even as low as five hours, it is obvious that the capacity to produce hypogean beans alone would favor the natural distribution of the hog peanut into lower latitudes far southward. As a matter of fact, its known distribution extends from New Brunswick and Minnesota to Nebraska and southward to Florida and the Gulf States.

It may be remarked that the flowering behavior of the hog peanut is quite comparable to that of the cultivated peanut (*Arachis hypogaea* L.). In the case of the latter, however, only those blossoms that can push themselves under the soil produce hypogean legumes or pods. Showy aerial flowers may arise from the higher leaf axils, but these never produce aerial pods as in the case of the hog peanut.

A number of familiar wild plants have a common habit of producing cleistogamic flowers with a more or less definite seasonal incidence. Among these are Venus lookingglass (*Specularia perfoliata* (L.) A. DC.), many violets, certain species of *Oxalis*, which regularly develop special subterranean stems late in summer, *Polygala polygama*, and the beautiful *P. pauciflora*. Beechdrops (*Leptamnium virginianum* (L.) Rab.) likewise develop chasmogamic and cleistogamic flowers at rather definite times during the season. In practically all the plants mentioned, the conspicuous or chasmogamic flowers may be entirely sterile or produce seed only occasionally. As a rule the cleistogamic flowers are highly fertile.

As in the case of the hog peanut, it will probably be found that the behavior of many wild plants such as those mentioned, expressing itself in the production of several types of flowers, varying from the conspicuous chasmogamic forms to the extremely reduced cleistogamic forms, represents definite responses to varying lengths of day. To say the least, length of day should now be looked upon as one of the seasonal factors which can in some instances exert a profound influence not only upon the initiation of flowering itself but upon the type of flower and inflorescence produced.

#### SUMMARY

Experiments have been carried out with the hog peanut (*Falcata comosa* (L.) Kuntze), giving it regulated exposures to daylight by means of suitable dark houses to exclude the daylight of early morning and late evening. Response to these conditions indicates that the blue aerial perfect flowers can develop only when the days are not less than 13½ hours long. The greenish aerial cleistogamic flowers can develop under all day lengths ranging from 5 to 13½ hours. Control plants (exposed for full day) usually did not develop any form of aerial flower until late July or August. The extremely rudimentary cleistogamic type of flower borne on the slender, filiform, nearly leafless stems and giving rise to hypogean pods was able to develop under all lengths of day from 5 hours up to 13½ hours. It is evident that the upper limit inhibiting this development lies

somewhere between  $13\frac{1}{2}$  and 15 hours, the maximum length of day of the Washington region. This is indicated by the fact that control plants did not produce these hypogean flowers until late July or early August.

The use of weak electric light to supplement the short days of winter from sunset until midnight inhibited the development of the winter form of aerial cleistogamic flowers. It did not entirely inhibit the development of filiform stems bearing the extremely rudimentary hypogean flowers when the additional light was withheld until 17 days after germination. In this instance no hypogean beans developed, and the filiform stems finally died. In a later test, when the additional light was afforded the plants from germination, the development of filiform stems was entirely inhibited.

In the field length of day seems to operate in fixing the more or less regular seasonal incidence of the several forms which appear to be derived from the normal blue flowers of the species, by gradations of reduction of the floral structures until the extreme hypogean type is attained.

These responses under controlled conditions indicate that the seasonal factor of length of day must be considered a potent influence in determining the kind of flowers that the hog peanut will produce. While the form of the hypogean bean is determined entirely by specific conditions associated with a soil environment, a specific length of day determines the particular type of axillary stem growth and geotropic behavior that will allow the rudimentary flowers to attain this particular environment.



# THE DOWNY SPOT DISEASE OF PECANS<sup>1</sup>

By J B DEMAREE, *Pathologist*, and J R COLE, *Associate Pathologist*, *Division of Horticultural Crops and Diseases*, *Bureau of Plant Industry*, *United States Department of Agriculture*

## INTRODUCTION

In 1927 Boyd<sup>2</sup> reported observing in southern Georgia a new foliage disease of the pecan (*Hicoria pecan* Brit.) and suspected that the causal fungus was a species of either *Cylindrosporium* or *Cercospora*. The same year Demaree and Cole<sup>3</sup> published a short description of the disease and considered the causal organism identical with *Cylindrosporium caryigenum* Ell and Ev. This fungus was first reported by Ellis and Everhart<sup>4</sup> from specimens of *H. cordiformis* (Wang.) Brit. (*Carya amara* Nutt.) collected by John Dearness near London, Ontario, in 1889. The fungus was later transferred to the form genus *Cercospora* by Hohnel<sup>5</sup>. The discovery of the perfect form, recorded in this paper, has compelled a change of the generic name, the name of the fungus now becoming, as will be shown, *Mycosphaerella caryigena* (Ell and Ev.) n. comb.

The disease was not noticeably abundant in southern pecan orchards in 1926, but the following year it became epiphytotic in restricted areas in southern Georgia and northern Florida. In 1928 it was less widely distributed, but probably was more abundant than in 1926. Because the disease is not a conspicuous one and is not especially destructive in Georgia, it may have been present there in pecan orchards for a number of years without being recognized as a distinct disease. Since 1926 it has been found over a large area in the southeastern part of the United States, including portions of Georgia, Florida, Alabama, Mississippi, Louisiana, Arkansas, and Texas, and furthermore it has been found to be more destructive in restricted localities of the drier regions of Louisiana and Texas than elsewhere.

## ECONOMIC IMPORTANCE

Since the disease has been under observation only a comparatively short time, its future economic importance can only be conjectured. There was a marked deficiency of rainfall in southern Georgia during the season of 1927, the year of the greatest observed prevalence of the disease, and future observations may show some association between its prevalence and a weakened condition of the trees caused by a deficiency of soil moisture, soil fertility, etc.

Observations made so far indicate that the disease may be a difficult one to control should it later prove to be destructive. It devel-

<sup>1</sup> Received for publication July 29, 1931, issued March, 1932.

<sup>2</sup> BOYD, O. C. PROGRESS REPORT ON THE EXPERIMENTS IN THE CONTROL OF PECAN SCAB AND LEAF CASE-BEARER AND ON THE OCCURRENCE OF AN UNDESCRIBED LEAF-SPOT OF PECANS. Natl. Pecan Growers Assoc. Proc. 26: 30-47. 1927.

<sup>3</sup> DEMAREE, J. B. and COLE, J. R. TWO UNREPORTED LEAF SPOTS OF PECAN. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Rpt. 11: 135-136. 1927. [Mimeographed.]

<sup>4</sup> ELLIS, J. B. and EVERHART, B. M. NEW SPECIES OF NORTH AMERICAN FUNGI FROM VARIOUS LOCALITIES. Phila. Acad. Nat. Sci. Proc. 1893: 168. 1894.

<sup>5</sup> HOHNEL, F. BEITRAG ZUR KENNNTNIS DER GATTUNG CYLINDROSPORIUM GREV. Ann. Mycol. 22: 199. 1924.

oped unabated in 1927, 1928, and 1929 in orchards where four and six applications of 20 per cent monohydrated copper sulphate and 80 per cent lime dust were used for the control of pecan scab. As far as the writers are aware, no experiments have been conducted with the primary object of controlling the disease, but observations on control have been made in spraying experiments conducted for the control of other pecan diseases.

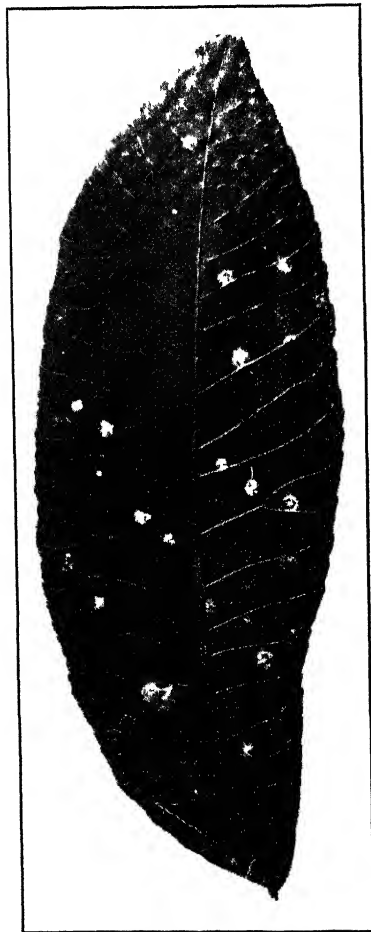


FIGURE 1.—White spots on the dorsal surface of a pecan leaflet, formed by the conidia of the downy-spot fungus

The fungus invasion does not kill the affected leaf tissues at first, but apparently it does destroy the chlorophyll in the affected areas, judging from the change of color from green to yellow. Consequently, numerous infections undoubtedly result in reduced photosynthesis. The disease has never been known to cause defoliation during the summer, but badly affected leaves ordinarily fall earlier than healthy ones.

There is some difference in the susceptibility of pecan varieties to parasitism by the fungus. The Delmas variety has shown greater susceptibility than others. This variety, however, formerly much planted, is not now considered commercially valuable within the region in which downy spot has been found, on account of its susceptibility to another pecan disease, pecan scab. The Moneymaker and Stuart, both important commercial varieties, rank next in susceptibility to downy spot. Of the other widely planted varieties the Frotscher has exhibited moderate susceptibility, while Schley, Alley, Success, and Pabst seem to be quite resistant.

#### DESCRIPTION OF THE DISEASE

The disease first appears on young pecan leaves during late spring or early summer. The first manifestation is the appearance of small, faintly marked, whitish spots on the underside of the leaves. These spots are made up of numerous minute clusters of conidia issuing from the stomata. The conidia are produced in great numbers from each stomatal opening on affected areas, and as the clusters enlarge they unite, forming distinct white spots from 2 to 5 mm in diameter (Fig. 1.) The action of dew sometimes spreads the conidia over or to one side of the affected host tissues as a thin white layer. Heavy rains wash away the conidia, leaving only slight evidence of the affected spots. At first the host tissues show little or no discoloration as a result of the para-

sitism As the infections become older the affected tissues turn yellow or light brown and are discernible also on the upper side of the leaves. The signs of the disease are never especially conspicuous after the conidia disappear. The white spots, while not permanent, furnish the most striking and descriptive sign of the presence of the pathogene.

On account of the yellow spots formed by the invasion of this *Cercosporella*, Boyd gave to the disease the name yellow leaf spot. Since the puncture of the pecan black aphid (*Myzocallis fumipennellus* Fitch) causes an even more conspicuous yellow spotting of pecan leaves, the writers feel that the name yellow leaf spot would frequently be confused with the effect of the aphid punctures, and therefore they suggest the name downy spot disease.

## THE CAUSAL FUNGUS

### CONIDIAL STAGE

The mycelium, although abundant, is confined principally to the spongy parenchyma tissues, but penetrates slightly into the palisade region. The hyphae seem to be intercellular only. During the early stage of the development of the fungus, subepidermal stromata are formed immediately below stomatal openings lying within infected areas. From the subepidermal stromata strands of hyphae extend through the stomatal openings (fig. 2, A) and form ectodermal stromata, from which numerous conidia are produced. There is some evidence that decumbent hyphae are formed from the outer stromata, from which conidia are also produced. The conidia are hyaline, mostly sickle shaped, pointed at the apex, and 2 to 3 septate. (Fig 2, B) Definite conidiophores have not been demonstrated. It is certain, however, that if the conidia are not sessile they must be formed on very short stalks.

Through the kindness of John Dearness, a cotype collection of *Cylindrosporium caryigenum* was examined by the writers. The conidia of the Canadian hickory specimens are formed from tufts of hyphae issuing from the stomata as described above for the form on the pecan, and there is no morphological difference between the conidia of the two forms except that those on the hickory are slightly smaller. Ellis and Everhart reported dimensions of conidia in the Dearness collection as  $25\mu$  to  $40\mu$  by  $3\mu$ , and Hohnel reported them as  $25\mu$  to  $46\mu$  by  $2\mu$  to  $3\mu$ . Conidia from the pecan range from  $25\mu$  to  $55\mu$  by  $4\mu$  to  $7\mu$ , being slightly longer and wider than those on the hickory.

### PYCNIDIAL STAGE

During late summer small conical pycnidiumlike structures (fig 2, D),  $43\mu$  to  $50\mu$  wide, form on the affected tissues that produced conidia earlier in the summer. These bodies are at first subepidermal and brown, but turn very dark brown or black after they break through the epidermis. The body wall is two to three cells thick. These structures are filled with minute rod-shaped, sporelike bodies, which escape through either a circular pore or a narrow slit. These bodies, which may be considered as pycnospores, microconidia, or spermatia, are hyaline and nonseptate. Repeated attempts to induce them to germinate in water have given negative results.

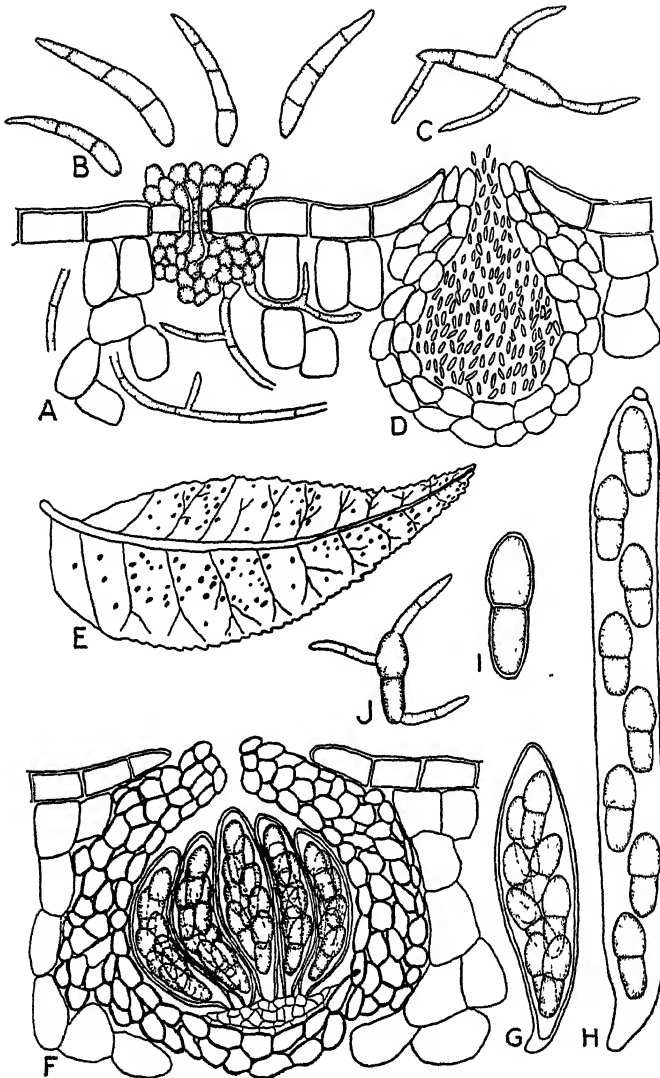


FIGURE 2—Conidial and ascigerous stage of the pecan downy spot fungus, *Mycosphaerella caryigena*. A, Cross section of pecan leaflet showing stomata above and below a stoma connected by hyphae,  $\times 500$ . B, Conidia,  $\times 140$ . C, Germinating conidium,  $\times 440$ . D, Pyrenidium containing sporelike bodies,  $\times 500$ . E, Pecan leaflet showing distribution of perithecia,  $\times \frac{1}{2}$ . F, Cross section of a mature perithecium,  $\times 650$ . G, Greatly enlarged ascus,  $\times 800$ . H, Ascus showing shape and arrangement of spores after elongation,  $\times 800$ . I, Enlarged ascospore,  $\times 1,500$ . J, Germinated ascospore  $\times 850$ .

## PERITHECIAL STAGE

Primordia of perithecia develop among the pycnidia during the latter half of the summer or early fall. These bodies are at first similar to the pycnidia in color and shape, but are larger and have either a flat or a rounded top.

The perithecia may develop in groups or in irregular rings surrounding the infected host tissues that previously produced *Cercospora* conidia (Fig 2, E). Those formed on the periphery of areas that produced conidia are larger than those found directly upon the lesions. If numerous infections of the *Cercospora* stage appeared on the leaves during the previous summer, the perithecia develop more or less uniformly over the entire lower surface of the affected leaves. The primordium of the perithecium appears first as an undifferentiated mass of hyaline fungus cells devoid of any evidence of a perithecial wall. The perithecial wall, which is formed during a later stage in the development of the fruiting body, is composed of several layers of thick-walled cells. The ostiole is a mere opening without any suggestion of a beak (Fig 2, F). Mature asci have been found on fallen leaves during March and April in orchards in southern Georgia, but asci developed more rapidly on leaves that were brought into the laboratory during early winter and placed in moist chambers.

The asci are hyaline, short, thick, and slightly clavate, and are packed closely within the perithecium. When removed from the perithecium and placed in water for microscopic examination they frequently elongate to twice their original length, or more (Fig 2, H). Prior to this elongation of the asci the eight spores are packed closely within the confines of the ascus wall (fig 2, G), but after elongation the spores rearrange themselves so as to occupy uniformly the increased space formed. The spores are liberated through a pore at the apex of the ascus after elongation of the ascus takes place. They are hyaline, 1 septate, and the two cells are slightly unequal in size (Fig 2, I). The smaller cell of the two points toward the base of the ascus.

## PATHOGENICITY AND RELATIONSHIP OF THE DIFFERENT FORMS

The discovery of perithecia and ascospores on fallen pecan leaves in districts where *Cercospora caryigena* was prevalent during the previous summer suggested a possible relationship between the two. Primordia of perithecia were observed developing on lesions caused by *C. caryigena* during late summer prior to leaf fall. Development of these primordia was followed through to maturity by frequent macroscopic and loose-mount examinations and by the aid of stained slides made at intervals during the development of the perithecia.

During the fall of 1927 pecan leaves were collected from a locality where the downy spot disease was abundant. At the same time other leaves were collected in a different orchard where the disease was not found. Both lots of leaves were kept out of doors during the winter in a coldframe covered with wire netting. When they were examined the following spring it was found that the collection of leaves bearing lesions of *Cercospora caryigena* produced abundant perithecia of the type described herein, and those leaves not having lesions of *C. caryigena* produced no perithecia of that type.

Artificial cultures made from ascospores make a growth indistinguishable from that made from conidia of *Cercospora caryigena*. Furthermore, similar conidia are produced in cultures made from either ascospores or conidia isolated from leaves. The pycnidia are considered to be related to the other two spore forms because of their frequent presence on lesions caused by the conidial stage, and because they are commonly produced in artificial cultures originating from either ascospores or conidia.

To obtain further evidence of the relationship of the perfect form to *Cercospora caryigena*, inoculation experiments were made. The following series of experiments were conducted in a locality where the *Cercospora* was not abundant.

1 Ascospores were collected by inverting plates of agar about 2 to 3 mm above leaves containing mature ascospores lying on moist filter paper. The spores were discharged on the surface of the agar and allowed to germinate. Blocks of agar containing the germinating spores were removed from the Petri dishes and placed on the under surface of young pecan leaves.

2 With the aid of a binocular microscope mature perithecia were removed from the leaf tissues, crushed, and placed in a watch glass containing about 10 c c of distilled water. A medicine dropper was used to place the inoculum suspension on the under side of young leaflets.

3 Conidia taken from the successful inoculations of Nos 1 and 2 were planted on agar plates and allowed to germinate. Blocks of agar with the germinating conidia were placed on young leaflets of the Moneymaker variety.

4. Conidia in water suspensions were applied to the under surface of young leaves with a medicine dropper.

One hundred inoculations were made in each series. Inoculated leaflets were inclosed in paraffin paper bags for a period of 24 hours. All inoculated leaflets were tagged and marked with powdered white crayon. No natural infection was found in experimental trees until after August 1, and not more than 1 per cent of noninoculated leaves were infected after that date. The results are set forth in Table 1.

TABLE 1.—Results of inoculation of pecan leaflets with conidia of *Cercospora caryigena* and the associated perfect stage \*

Inoculum	Date of inoculation	Date record taken	Number of inoculations	Number of infections
Germinated ascospores in cubes of agar.....	Apr 28	June 16	100	31
Do.....	May 7	June 25	100	37
Macerated perithecia in water suspension.....	do.....	do.....	100	5
Germinated ascospores in cubes of agar.....	June 1	July 10	100	55
Do.....	June 10	July 20	100	43
Macerated perithecia in water suspension.....	do.....	do.....	100	10
Germinated conidia in cubes of agar, originating from infections by ascospores.....	July 15	Aug 20	100	63
Do.....	July 20	Aug 30	100	30
Conidia in water suspension, taken from successful ascospore inoculations.....	Aug 1	Sept 15	100	0

\* The inoculations reported were performed by the junior writer near Shreveport, La.

Of the 600 ascosporic inoculations made, 181 resulted in infections typical of *Cercospora caryigena*. A period of six to seven weeks

was required from the time the inoculum was applied to the leaves until *Cercospora* conidia were produced. Conidia that developed from ascospore inoculations were used as the inoculum for subsequent experiments, which in turn produced *Cercospora* conidia. The largest percentage of successful inoculations resulted from germinated ascospores and conidia in agar. Inoculations from macerated perithecia gave a low percentage of infection, but no infections resulted when the inoculum consisted of conidia in water suspension.

As a result of the cultural and inoculation experiments, the relationship of the *Cercospora* stage and the *Mycosphaerella* stage is proved, and both are shown to be pathogenic.

#### TAXONOMIC POSITION AND TECHNICAL DESCRIPTION

The characters of the ascigerous stage of this fungus correspond to those of the genus *Mycosphaerella* as described by Johanson, to which genus this fungus is assigned. Apparently there has not been described a *Mycosphaerella* on the pecan or other species of *Hicoria* that is morphologically identical with this one, and since it has proved to be connected with *Cercospora caryigena* (Ell. and Ev.) Hohnel, it may appropriately be given the name *Mycosphaerella caryigena* with the following description:

##### *Mycosphaerella caryigena*, n. comb.

SYNONYMS *Cylindrosporium caryigenum* Ellis and Everhart Proc. Acad. Nat. Sci., Phil., Pa., 1889

*Cercospora caryigena* (Ell. and Ev.) Hohnel, Ann. Mycol., vol. 22, 1924

ASCIGEROUS STAGE.—Perithecia hypophyllous, black, crumpled, aggregate to scattered, ellipsoid-globose,  $50\mu$  to  $85\mu$  in diameter, ostiolate. Perithecial wall 2 to 5 cells thick. Asci clustered, clavate-cylindrical, hyaline, 8-spored,  $45\text{--}55\mu$  by  $7.5\text{--}9\mu$  (often becoming much longer when placed in water), no paraphyses, spores hyaline, 1-septate, slightly unequal,  $12\text{--}15\mu$  by  $3\text{--}5\mu$ .

CONIDIAL STAGE.—Hyphae principally confined to spongy parenchyma, conidia profuse, formed from tufts of hyphae penetrating through stomata, hyaline, filiform, curved, 2 to 3 septate,  $25\text{--}55\mu$  by  $4\text{--}7\mu$ . Conidia form white spots on lower leaf surface of *Hicoria*, later disappearing, and lesions show as moderately conspicuous yellow to light-brown spots 2 to 5 mm in diameter.

PYCNIDIAL STAGE.—Pycnidia appear during late summer on lesions caused by the *Cercospora* stage, hypophyllous, first brown, later black, crumpled,  $45\text{--}50\mu$  wide. Pycnospores rod-shaped, hyaline, nonseptate,  $2\text{--}3.5\mu$  by  $1\text{--}1.5\mu$ . Function unknown.

Parasitic on leaves of *Hicoria* species.

Ascigerous stage first appearing on leaves of *Hicoria pecan* during late summer; development completed in March and April of following year.

#### PHYSIOLOGICAL STUDIES ON ARTIFICIAL MEDIA

Monosporous cultures from conidia were readily made by removing them with a sterile needle from young sporulating lesions and "streaking" agar plates. Isolated conidia were later transferred to agar slants. The perfect stage was cultured by inverting plates of agar over fragments of leaf tissue containing mature perithecia. The most satisfactory plantings were made when the perithecia were lying about 2 mm below the surface of the agar. Isolated spores were marked, and those that germinated were transferred to tubes containing agar. The cultures grew very slowly at first, requiring about eight days at room temperature (about  $23$  to  $27^{\circ}\text{C}$ ) to become macroscopic in size, and attained a diameter of 1 to  $1\frac{1}{2}$  mm in about two weeks.

The fungus was grown on the following types of artificial culture media: Beef agar, corn-meal agar, Lima-bean agar, dextrose agar, a mixture of corn-meal and potato agar, pecan leaves, watermelon rind, and bean pods.

The reaction of the fungus on each medium used was different. On dextrose agar (Difco) it produced a dark-brown to black granular thick stroma on the surface of the medium. Little or no aerial mycelium was present. The advancing portions of the stroma had a creamy to grayish color and were somewhat slimy. The entire stroma as seen through a lens had a wet, slimy appearance. On Lima-bean agar the exposed stroma was rough or granular and dark brown. The stromata were usually covered with a mat of white hyphae, surrounded by white to creamy, slimy, submerged portions.

Cultures on most media form conidia and pycnidia in abundance. Both conidia and pycnospores closely resemble those occurring naturally on the host. Perithecia have not been known to form on artificial media.

On artificial media the fungus made the most vigorous growth at temperatures ranging from 23° to 27° C. Growth is inhibited at a temperature of about 5°, but is resumed when the temperature becomes more favorable.

The fungus was grown at the optimum temperature for 60 days on corn-meal agar, having a hydrogen-ion concentration of pH 3.8 to 8.5. The best growth was made at pH 6.5. Below pH 4.5 and above 8.0 growth was very meager.

#### SUMMARY

The conidial stage of the downy spot disease of pecans is considered identical with *Cercospora caryigena* (Ell. and Ev.) Hohnel, a fungus first described by Ellis and Everhart as *Cylindrosporium caryigenum* on specimens of *Hicoria cordiformis* collected by John Dearness near London, Ontario, in 1889.

Conidia are produced in great numbers on lesions and form white spots 2 to 5 mm. in diameter on the lower surface of affected leaves. When the conidia are washed off by rains the lesions appear as inconspicuous yellow or brown spots. The disease has been observed on the pecan in Georgia, Florida, Alabama, Mississippi, Louisiana, Arkansas, and Texas.

The perfect stage, *Mycosphaerella caryigena* n. comb., develops on pecan leaves during fall and winter, but does not mature until early spring. Proof of the relationship of the conidial and perfect stages and of their pathogenicity was demonstrated by comparison on artificial media and by inoculation experiments.

A description of the fungus and its growth on culture media is given.

# PHYSIOLOGIC RACES OF *USTILAGO LEVIS* AND *U. AVENAE* ON RED OATS<sup>1</sup>

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## INTRODUCTION

The results of a study of smut specimens for the determination of specialized races of *Ustilago avenae* (Pers.) Jens. and *U. levis* (Kell. and Sw.) Magn. are presented in the following pages. This paper also reports the identification of a hitherto unknown physiologic race of *U. levis* which attacks Fulghum oats.

## REVIEW OF LITERATURE

Extensive data on physiologic races of the fungi that cause loose and covered smuts on oats have been published by Reed (4, 5, 6)<sup>2</sup> and by Sampson (7, 8). Several distinct races of both species have been identified. Among the races of *Ustilago avenae* two of particular interest attack the Fulghum and Red Rustproof varieties. The Fulghum race of *U. avenae* is capable of severely smutting Fulghum and the closely related Kanota and Frazier strains. In addition, several varieties of common oats, such as Bicknell, Black Diamond, Canadian, and Victor, are severely attacked. Hull-less or naked oats and the wild species *Avena barbata* Brot. also are very susceptible. On the other hand, strains of the Red Rustproof type are extremely resistant to this particular race of *U. avenae*.

The physiologic race of *Ustilago avenae* which attacks Red Rustproof also is highly specialized. It produces a large percentage of smutted plants on Red Rustproof and the related Nortex. On Fulghum and its strains, however, it gives essentially negative results. Apparently there is only one variety of the *Avena sativa* group, namely Canadian, that is susceptible. Hull-less oats also are extremely resistant. It is an interesting fact, however, that *A. barbata* is completely susceptible.

Hitherto no race of *Ustilago levis* has been known to attack seriously Fulghum and Red Rustproof oats. Reed (6) has shown that Fulghum occasionally may be slightly smutted by a form of *U. levis* from Missouri, but the percentage of smutted plants is never very high. Occasional plants of Red Rustproof, apparently smutted with this same race, also have been found.

## IMPORTANCE OF FULGHUM AND RED RUSTPROOF OATS

Various named strains of the Fulghum and Red Rustproof varieties of oats are grown extensively in the southern half of the United

<sup>1</sup> Received for publication July 17, 1931, issued March, 1932. The investigations here reported were conducted by the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, in cooperation with the Brooklyn Botanic Garden, Brooklyn, N. Y. Contribution No. 62 Brooklyn Botanic Garden.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 152.

States In the Cotton Belt they are grown almost exclusively, and mostly from fall seeding In the region immediately to the north of the Cotton Belt they are important spring-sown varieties, especially Fulghum and its strain Kanota Fulghum is now most extensively grown in Missouri, Kansas, and Oklahoma, where it has largely replaced the Burt and Red Rustproof varieties The last named is not altogether satisfactory for spring seeding, owing to its later maturity Prior to the development of Fulghum, early varieties of common oats such as Kherson and Sixty-Day also were grown to some extent in this area, with generally unsatisfactory results, owing to poor adaptation Stanton and Coffman (9, 10) have made available information on the importance and distribution of the Fulghum and Red Rustproof varieties

### MATERIALS AND METHODS

In the spring of 1929 the junior writer collected samples of oat smut in various parts of the South and forwarded them to the senior writer at the Brooklyn Botanic Garden An identification number was assigned to each collection, by which each is hereafter designated Ten of these collections have been tested for their physiologic specialization. The oat varieties used as testers or differential hosts were Canadian (seed No.<sup>3</sup> 119), Victor (seed No 126), Fulghum (seed No 129), Red Rustproof (seed No 131), and Navarro (Ferguson Navarro; seed No. 939, C I.<sup>4</sup> No 966)

The inoculations were made by the method described by Reed (6). All tests were made under greenhouse conditions at the Brooklyn Botanic Garden

### EXPERIMENTAL DATA

The reaction of the five differential hosts to *Ustilago levis* collected from the Fulghum variety, is shown in Table 1 Collection numbers and geographic origin are likewise shown

TABLE 1—Reaction of the five differential hosts to *Ustilago levis* collected from Fulghum oats

Variety	Collection No 16, Athens, Ga			Collection No 18, Clemson College, S C		
	Plants		Infected	Plants		Infected
	Number	Number		Number	Number	Per cent
Canadian.....	13	13	100 0	17	17	100.0
Victor.....	19	16	84 2	19	19	100 0
Fulghum.....	19	18	91 7	19	15	78 9
Red Rustproof.....	19	0	0	18	0	0
Navarro.....	19	0	0	19	0	0

Collections Nos 16 and 18 proved to be typical of the covered smut. Both produced high percentages of smut on Fulghum Collection No 16 produced smut on 18 of the 19 plants, or 94 7 per cent, and collection No 18 produced smut on 15 of 19 plants, or 78.9 per cent. Collection No. 16 gave 100 per cent infection on Canadian and 84.2

<sup>3</sup> Seed numbers designate special strains of the varieties propagated and maintained by the senior writer  
<sup>4</sup> C I refers to accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations

per cent on Victor, while collection No 18 produced 100 per cent infection on both of these varieties. Red Rustproof and Navarro proved highly resistant to both collections

The reaction of the five differential hosts to *Ustilago avenae* collected from the Norton, Kanota, and Frazier varieties (collections Nos 8, 48, and 56, respectively) is shown in Table 2. The geographic origin of each collection is also shown.

TABLE 2—*Reaction of the five differential hosts to Ustilago avenae collected from Norton, Kanota, and Frazier oats*

Variety	Collection No 8, A and M College, Miss			Collection No 48, Near Newton, Kan			Collection No 56, Lawton, Okla		
	Plants		Infected	Plants		Infected	Plants		Infected
	Number	Number		Number	Number		Number	Number	
Canadian .....	19	19	100 0	19	17	89 4	20	20	100 0
Victor .....	20	8	40 0	20	11	55 0	18	17	94 4
Fulghum .....	20	20	100 0	20	20	100 0	20	20	100 0
Red Rustproof .....	19	0	0	19	0	0	20	0	0
Navarro .....	20	0	0	20	0	0	20	0	0

Collection Nos 8, 48, and 56 (Table 2) correspond quite closely in reaction to the Fulghum race of *Ustilago levis* shown in Table 1. All three collections gave 100 per cent infection on the Fulghum variety. Very high percentages of smut also were obtained on Canadian. The Victor variety gave somewhat variable results, the percentage of smutted plants ranging from 40 0 to 94 4. The results with Red Rustproof and Navarro were entirely negative. These data are in harmony with those reported by Reed (5, 6) for the physiologic race of *U avenae* on Fulghum oats.

The reaction of the five differential hosts to *Ustilago avenae* collected from the Ferguson No. 922, Nicholson Hundred Bushel, and Nortex strains of the Red Rustproof variety (collections 25, 53, and 57, respectively) is shown in Table 3. The geographic origin of each collection is given in the table.

TABLE 3—*Reaction of the five differential hosts to Ustilago avenae collected from Ferguson No 922, Nicholson Hundred Bushel, and Nortex oats*

Variety	Collection No 25, Denton, Tex			Collection No 53, Stillwater, Okla			Collection No 57; Lawton, Okla		
	Plants		Infected	Plants		Infected	Plants		Infected
	Number	Number		Number	Number		Number	Number	
Canadian .....	19	16	84 2	20	19	95 0	19	17	89 4
Victor .....	20	0	0	20	2	10 0	20	1	5 0
Fulghum .....	20	0	0	20	0	0	20	6	30 0
Red Rustproof .....	20	15	75 0	20	14	70 0	20	19	95 0
Navarro .....	20	0	0	19	0	0	19	0	0

Collections Nos. 25, 53, and 57 are very similar to, if not identical with, the previously described form of *Ustilago avenae* obtained from Red Rustproof oats (5, 6). The three named strains of Red Rustproof from which the collections were made are typical of the Red

Rustproof variety. Comparatively high percentages of smutted plants were obtained with all three collections on Red Rustproof, the percentages ranging from 70.0 to 95.0. A somewhat higher percentage of smut (from 84.2 to 95.0 per cent) was secured with the variety Canadian. A few smutted plants of Victor were obtained with two of the collections, but no smutted plants whatever were obtained on Navarro. Fulghum gave negative results with two of the collections, but with No. 57, 30 per cent of the plants, or 6 out of 20, were smutted. There is a possibility in this case that the original collection of smut was a mixture of the Red Rustproof and Fulghum races. Further experiments are necessary to determine whether this is the case.

Two additional collections from Cowra No. 22 and Colburn (C. I. No. 2019) also have given interesting results. The reaction of the five differential hosts to *Ustilago avenae* collected from these varieties (collections Nos. 46 and 59, respectively) is shown in Table 4. The geographic sources of the collections are shown in the table.

TABLE 4.—Reaction of the five differential hosts to *Ustilago avenae* collected from Cowra No. 22 and Colburn oats

Variety	Collection No. 46, Experiment, Ga.			Collection No. 59, Lawton, Okla.		
	Plants		Infected	Plants		Infected
	Number	Number	Per cent	Number	Number	Per cent
Canadian.....	19	19	100	19	19	100
Victor.....	19	19	100	19	19	100
Fulghum.....	20	3	15	20	0	0
Red Rustproof.....	20	0	0	20	0	0
Navarro.....	20	0	0	20	0	0

Collection No. 46, from the Cowra No. 22 variety, gave 100 per cent infection on both Canadian and Victor. The Fulghum variety showed 15 per cent of smutted plants, 3 out of a total of 20 being smutted. Negative results were secured with Red Rustproof and Navarro. In its ability to infect Fulghum slightly and to infect Canadian and Victor heavily, this collection is allied to the Missouri race of *Ustilago avenae*. The remaining collection, No. 59, gave 100 per cent infection on the two varieties, Canadian and Victor, while entirely negative results were secured with Fulghum, Red Rustproof, and Navarro.

#### DISCUSSION

The most interesting feature of the results of this study is the identification of a definite specialized race of *Ustilago levis*, hitherto unknown, which attacks the Fulghum variety of oats (Fig. 1). The two collections, Nos. 16 and 18, are typical of this species, and both show a high degree of virulence for this type of oat. As yet no similar race of covered smut capable of infecting the Red Rustproof variety has been demonstrated. It is, however, very probable that such a form or race is actually in existence and will be identified sooner or later.

Further experiments are in progress to determine more definitely the extent of the specialization of the new collections. A large number

of varieties of oats, representing different types, have been inoculated in order to determine their susceptibility

Some further discussion of the relationship of such varieties as Norton, Cowra No 22, and Colburt to Red Rustproof and Fulghum in connection with their reaction to certain of the 10 smut collections reported in this paper seems desirable

It was to be expected that Norton would show high susceptibility to *Ustilago avenae* collected from Fulghum. Norton was originated as a selection from a cross between Fulghum (Coker Fulghum strain No 3) and an unnamed gray oat (R-F-3) with side panicle, which was obtained as an individual plant from a field of mixed Red Rust-

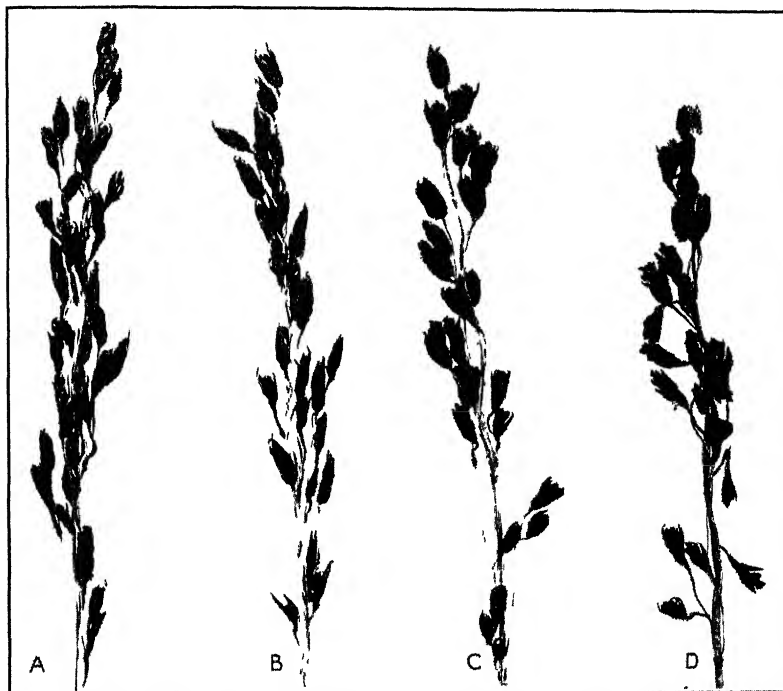


FIGURE 1 Smutted panicles of Fulghum oats, A and B infected with *Ustilago levis*, C and D with *U. avenae*. The contrast between the two smuts is very evident

proof by George J. Wilds, jr, in 1918. The cross was made a few years later by J B Norton in the breeding nurseries of Coker's Pedigreed Seed Co, Hartsville, S C.

The close relationship of Norton to the Kanota and Frazier strains of Fulghum is demonstrated by the results presented in Table 2. On the other hand, relative to plant characters, Norton resembles common rather than red oats. These facts undoubtedly furnish further evidence that the inheritance of susceptibility or resistance to smut in oats is not linked with morphological characters.

The Cowra No 22 (also known as Quandong) variety was introduced from Australia. According to Pridham (2, 3) it was originated as a selection from Ruakura on the Cowra Experiment Farm, New South Wales. The latter variety was developed from a plant varia-

tion from the Red Algerian (Argentina), a variety belonging to *Avena byzantina* C Koch, morphologically similar to the well-known Red Rustproof oat of the South Ruakura, however, usually has been classified as belonging to *A sativa* L. Cowra No. 22 is similar to Ruakura in that it is more or less intermediate in type between the varieties of *A sativa* and *A byzantina*. In this connection it is of interest to point out that differential hosts belonging to both of these groups reacted to the smut collected on Cowra No. 22 (Table 4.)

The origin of the Colburt variety has been reported by Stanton, Griffie, and Etheridge (11) and by Coffman (1). It was developed as a plant selection from Burt, a red oat, at Akron, Colo. However, Colburt is an early black common oat (*Avena sativa*), morphologically similar to Monarch. Colburt is a very uniform variety and evidently represents a mechanical mixture rather than a plant variation from Burt. As a consequence, Colburt probably is not closely related to such varieties as Fulghum, Red Rustproof, and Navarro. The data shown in Table 4 indicate specialization of the race of smut collected on Colburt to varieties belonging to *A sativa*. It is very probable, therefore, that this smut was introduced from Akron, Colo., to Lawton, Okla., on Colburt itself.

#### SUMMARY

Results of a study of a collection of smut specimens, mostly of red oats, for the determination of specialized races of *Ustilago avenae* and *U levis* are reported.

The identification of a hitherto unknown specialized race of covered smut which attacks Fulghum oats is demonstrated.

As red oats are grown extensively and in some sections almost exclusively in the southern half of the United States, the identification of a specialized race of covered smut attacking Fulghum may be of considerable economic importance.

Of the 10 collections studied, 2 were typical of *Ustilago levis*, and both showed a high degree of virulence in attacking Fulghum. As yet no similar race of covered smut capable of infecting the closely allied Red Rustproof variety has been identified.

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# BLACK SCORCH OF THE DATE PALM CAUSED BY *THIELAVIOPSIS PARADOXA*<sup>1</sup>

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## INTRODUCTION

A fungous disease of economic importance has recently appeared on the date palm, *Phoenix dactylifera*, in the Southwest. The fungus, *Thielaviopsis paradoxa* (De Seynes) Von Hohn,<sup>2</sup> has been found attacking all organs of the palm except the roots and stem, and these latter organs have been found susceptible by artificial inoculation. Successful infections by means of inoculations have been obtained on all parts of the date palm, and the organism in all cases has been readily reisolated. While the total losses from this disease up to the present are apparently of minor importance, the severity of its attack in some instances indicates that it may become so troublesome as to require special measures of control.

## DISTRIBUTION

In the so-called "bud-scorch" form of the malady is widely distributed, being present in every garden inspected in the Coachella Valley, Calif., and Arizona. It has also been found on ornamental date palms at Riverside, Calif. Other workers have found that the fungus parasitizes a number of plants, including areca palms, oil palms, sugarcane, coconut, and pineapple. Edgerton (3)<sup>3</sup> describes it as causing great damage to sugarcane. Although it has not been reported as occurring on Citrus, the writers have found that it produces a firm, dark, smoky-colored, pleasantly aromatic decay when introduced into wounds of citrus fruits. In India, Sundararaman, Krishnan Nayar, and Ramakrishnan (11) have shown experimentally that it is capable of attacking plantain, mango, *Saccharum spontaneum*, *Rhapis* sp., and the date palm. Except in the abstract by Klotz and Raby (8) and in papers by Fawcett (4) and Klotz (7), it is believed that the organism has never been reported as attacking the date palm naturally. On preserved specimens of apparently the same disease collected by Fawcett (4) in Egypt, Algeria, and Tunisia, the writers have found conidia typically like those of *Thielaviopsis*. How seriously the fungus attacks the inflorescences and lessens the quantity of fruit depends upon weather conditions preceding and during the time of emergence of the spathes. It is likely that mildly warm, moist weather accompanying or alternating with windy weather favors distribution and infection. The optimum temperature for growth of the fungus lies between 24° and 27½° C. The manner in which the conidia are borne (in extremely long chains, which readily break up into small groups and single conidia) favors distribution by wind. Germination of the spores on glass is possible only in the presence of water in liquid form.

<sup>1</sup> Received for publication Aug. 4, 1931, issued March, 1932. Paper No. 257, University of California Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside, Calif.

<sup>2</sup> Identified by J. A. Stevenson, of the Division of Mycology and Disease Survey, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 165.

## SYMPTOMS OF THE DISEASE

## ON SPATHE, FRUITSTALK, AND FRUIT STRANDS

As shown in Figure 1, the parasite attacks the young fruitstalks and fruit strands even before the spathe has ruptured. On the spathe, circular to elongated lesions mark the points of entrance of the disease. These lesions range in color from sorghum brown (Ridgway)<sup>4</sup> on the

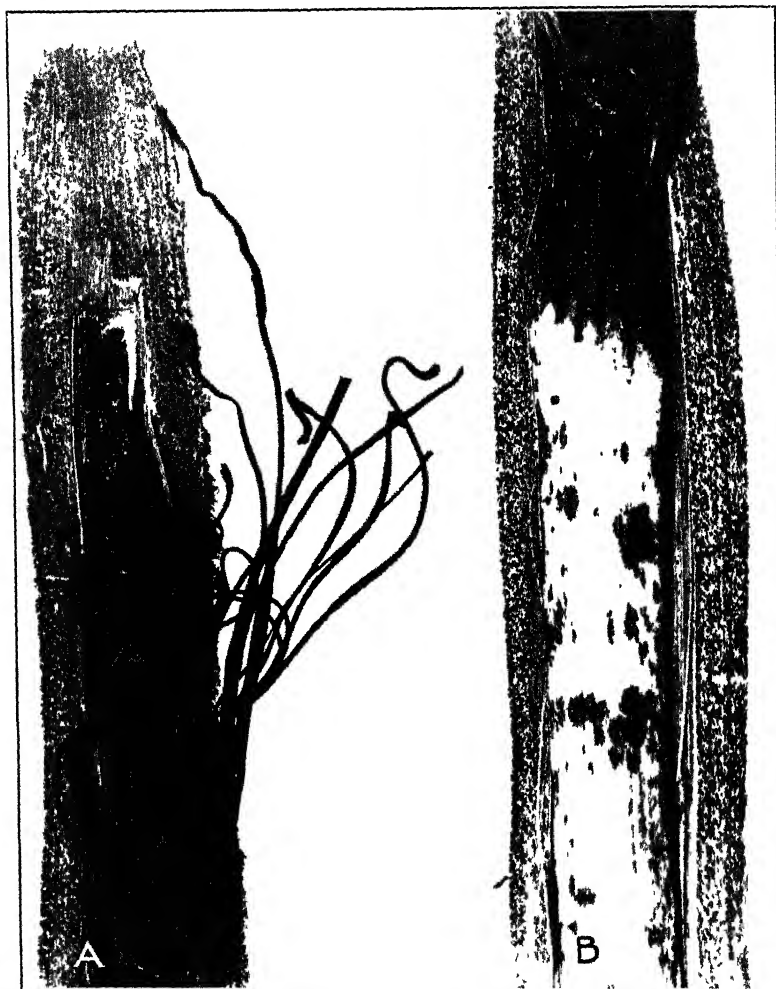


FIGURE 1.—Female spathe cut open to show (A) black scorch on inflorescence, the gnarled and twisted fruit strands being devoid of flowers, (B) lesions on fruitstalk

exterior surface to mahogany red or bay on the interior surface. On removal of a portion of the infected spathe it was found that the fruitstalk bore depressed, brown (warm blackish) to black necrotic areas, which were circular to oblong in outline. The twisted deformed fruit strands of the specimen shown in Figure 1, A, were entirely diseased

<sup>4</sup> RIDGWAY R. COLOR STANDARDS AND COLOR NOMENCLATURE 43 p., illus. Washington, D. C. 1912

They were blackish brown to black in color, and were devoid of flowers. Microscopic examination showed them to be covered by the typical dark-brown, unicellular, oval conidia. The strands of fruit bunches that were attacked later in their development showed blackened, depressed lesions (fig. 2) similar to those on the fruitstalk, and some were completely severed by the decay. The affected tissue was in all instances dry and firm and each area bore the black powdery spores. A gray covering on some of the lesions was found to be due to conidia of *Fusarium* sp. A species of *Fusarium* was later found as the primary cause of a decay of certain male inflorescences of the date palm. Inoculations by means of spore suspensions of *Thielaviopsis* into young spathes which were just beginning to crack open showed that wounding was unnecessary for infection, the typical dark lesions being produced on the young tender fruit strands and fruitstalks.

#### ON TERMINAL BUD

The effect of the disease on the palm bud and heart is even more serious than it is on the fruitstalks. The pathogene gains entrance to the succulent tissue through a wound or puncture, and its progress in this vital region is very rapid. The entire terminal bud and adjacent leaf bases may succumb, eventually presenting a dried, dull, blackened, charcoallike appearance. Two large date seedlings in boxes in the greenhouse were killed by inoculations at the base of the young central leaves. In four of the five cases observed in the field the entire bud was not killed but grew out laterally, producing the so-called "fool disease" effect (called by the Arabs "medjnoon"). It is believed that in California *Thielaviopsis paradora* is the principal organism causing this peculiar trouble. Eventually, the entire bud regenerates from the uninjured portions of meristematic tissue and returns to its normal vertical position. High temperatures and rapid growth of the palm may be the factors that operate to prevent the disease from terminating fatally in all instances. On laboratory media the fungus makes very little growth at 32° C. or above.

#### ON THE PETIOLE, MIDRIB, AND PINNAE

The blackening of the midrib of fronds that usually accompanies the bud-scorch form of the disease may frequently be due to the same organism. The black, irregular, rough, necrotic condition of the leafstalk (fig. 3) is the most striking symptom of the disease. It gives the impression that the tissues have been burned, and suggests the name selected for the disease—black scorch. The cross cuts and V cuts so commonly found near the base of a midrib present an ideal entrance for this and other fungi.

Ashby (1) and Orian (9) have reported the fungus as attacking the pinnae of the freshly opened leaves of coconut palm. "Pale yellow spots with a brown margin develop on the furled pinnae. Later the lesions elongate, converge, and turn black, owing to the presence in the tissues of spores of the fungus. Infection spreads rapidly through the pinnae, and in severe cases the heart leaves dry up." This, so far as it goes, is an accurate description of the course of the disease produced by the writers on a large seedling in the greenhouse. On this seedling and on material collected in the field, the midribs and pinnae had circular to elongated irregular spots, which in some in-

stances were as wide as the pinnae and as much as 5 centimeters in length. Artificial wounding was unnecessary to secure infection on the petiole, midrib, and pinnae. The fungus readily invaded the margin of the petiole where the fibers originate. Twenty days after



FIGURE 2—Black-scorch lesions on fruit strands  $\times \frac{3}{8}$

spores were placed on a leaf of a seedling palm, both edges of one petiole had lesions 3 to 10 millimeters in depth and 150 millimeters in length. (Fig 4, A.) In the chlorophyll-less region of the petiole

base, the lesion was yellow ocher in color,<sup>5</sup> and in the green region, Dresden brown. The outer margin of the lesion was dark brown to black, while the inner margin was a light chestnut brown. The central area



FIGURE 3 —Deformed young date fronds showing charcoal like effect that follows infection by black scorch, the fungus may accompany the deformity but does not necessarily initiate it

(17 by 10 millimeters) of a typical isolated spot (20 by 16 millimeters) on the dorsal surface of the same petiole (fig. 4, A) was chestnut brown, bounded by a narrow margin of deep brown to black. Black

<sup>5</sup> RIDGWAY, R. Op cit.

spore masses were scattered throughout this area. Surrounding the inner area was a band 2 to 3 millimeters in width and yellow ochre in color. The margin of the band was a light chestnut brown. Figure 4, B, shows typical spots on the pinnae. These ranged in size from microscopic to 20 by 8 millimeters. Their color characters were similar to those given for the spot on the petiole. However, as a lesion on a pinna dries, the chestnut-brown center gradually becomes lighter until it is a warm buff.

Several midrib bases of the second whorl of fronds on a large seedling offshoot were inoculated by placing the fungus in a 3-millimeter hole

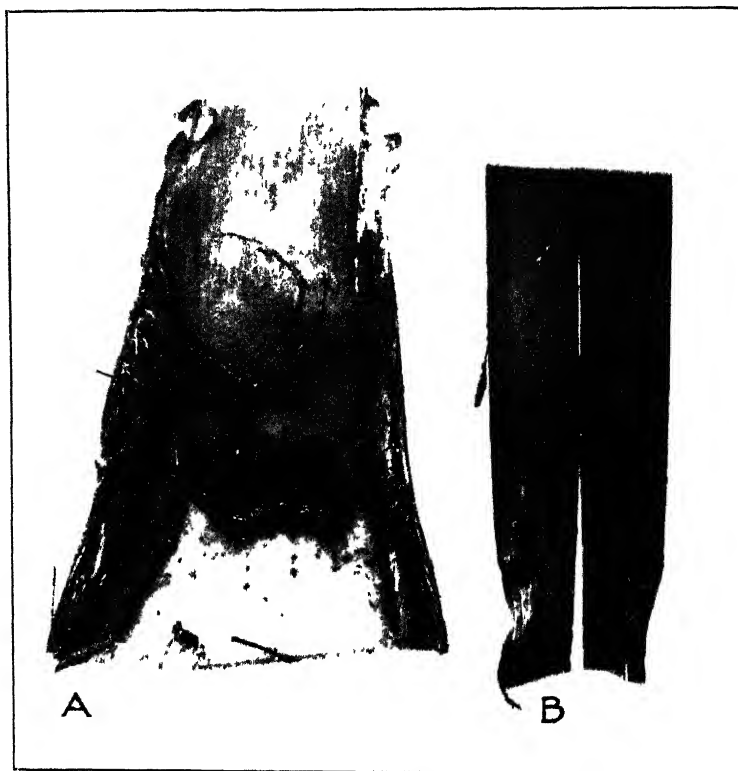


FIGURE 4—Lesions on petiole base (A) and pinna (B) produced by fungus in absence of wound. The petiole margins where the fibers originate should be considered as wounded tissue.

made with a cork borer. The wound was covered with adhesive tape until the organism had become established. In four weeks the fungus on one frond had produced an oval lesion 9 centimeters wide and 15 centimeters long, almost enveloping the midrib and causing it to break. Extending for a total length of 45 centimeters up and down the surface of the midrib beyond the lesion was a linear series of circular water-soaked areas each about 12 millimeters in diameter. The fungus was reisolated from the water-soaked area most remote from the point of inoculation, which shows that the organism invaded new tissue at the rate of at least  $1\frac{1}{2}$  centimeters per day for 30 days. The surface of the canker was gray to brown to drab in color, and the

pinnae beyond turned gray as they dried. Internally the lesion was light drab to wine in color toward the advancing edges, with smaller orange to reddish-brown streaks extending far up and down the midrib. (Fig. 5) These streaks were directly under the water-soaked areas that appeared on the surface. The pathogene was readily reisolated from any portion of the affected tissue. Eventually the invaded tissue turned black owing to the production of fuscous spores by the causal fungus. Likewise, inoculations of any pruning cuts and the cut surfaces of midribs and spines were invariably successful, the infected tissue dying back several inches and eventually becoming blackened and covered by fungous spores.

#### ON STEM AND ROOT INDUCED BY ARTIFICIAL INOCULATION

To test the susceptibility of the trunk or stem of date palm to the fungus, the old leaf bases were cut away, the surface cleaned with alcohol, and a portion of an agar culture inserted in a hole made with a quarter-inch cork borer. The inoculation was covered with adhesive tape. Five weeks later an examination revealed a zone of dead and dried brown tissue extending in all directions from the point of inoculation. The dark spores of the fungus were present in this region. Beyond the dead tissue was a narrow, pinkish zone about 6 millimeters in width, and beyond that a tumeric-yellow band about 25 millimeters wide. The diseased tissue extended 7 to 10 centimeters from the focus of infection. It was brown to drab in color beyond the yellow zone and had no well-defined margin. It is difficult to determine the extent of diseased areas because the excised tissue darkens rapidly in the air.<sup>6</sup>

Roots of the date palm were likewise tested. On the northwest side of a seedling palm, 15 roots about 12 millimeters in diameter were carefully uncovered. Eight of these were inoculated; some by simply placing agar inoculum on the unbroken surface, and others by inserting the fungus in a 12-millimeter longitudinal slit made with a scalpel and covering the place of inoculation with moist cotton and waxed paper. All of the inoculated roots decayed, the affected portions being a soft, moist, brown decay which became a darker brown as the fungus fruited. In five weeks the affected tissue extended from 5 to 15 centimeters in both directions from the point of inoculation along the root. The inoculated roots, wounded and unwounded, showed no decay. The organism was reisolated from the diseased stem and roots.

#### VARIETAL SUSCEPTIBILITY

The midrib-scorch form of the disease has been found on all varieties of date palms growing in the Southwest, except the Tazizaoot. Although the fungus was first found causing inflorescence decay on the Deglet Noor, this variety in the Coachella Valley is perhaps one of the least susceptible to other forms of the disease. The Thoory variety appears to be very susceptible to the midrib-scorch form of the malady. The fibers of the midribs of the outer whorls of leaves seem to bind, and as growth proceeds from the center, to injure the young emerging fronds, thus affording an excellent opportunity for infection.

<sup>6</sup> R. B. Streets, of the University of Arizona, at the 1930 Date Growers' Institute reported orally on a disease of the stems of neglected date palms in Arizona having symptoms similar to those on trunk tissue described here. The name of the organism was not mentioned at the time, but later it was identified as a species of *Thielavia* (8).

by the black-scorch fungus, even in the absence of moisture. Winds probably accentuate this type of mechanical injury. The cross cuts

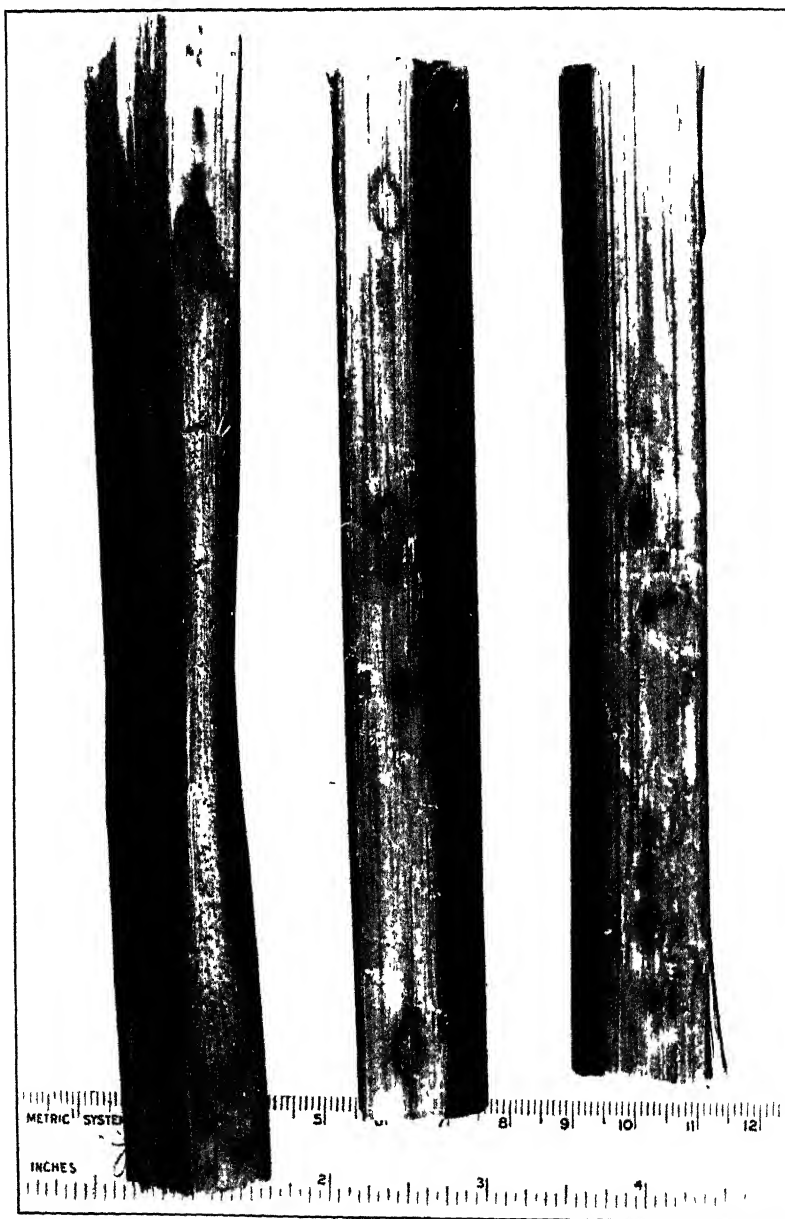


FIGURE 5—Diseased internal tissues of petiole produced by artificial inoculation with the black-scorch organism

and V cuts mentioned earlier probably have the same origin. The abundance of the disease on the Thoory variety may be due to the greater incidence of this type of injury on that variety.

The Hayany, Amhat, Saidy, and Halawî varieties are likewise very susceptible, perhaps more so than the Deglet Noor. The disease was observed also on the Zahcedy, Menakher, Baklany, Guntar, Haloo, Fteemy, Besser Haloo, Nakleh-Zian, Sukar-Nabat, Horra, and and Koroeh, but the number of individuals was too small to permit reliable comparison.

#### THE PATHOGENE

Patterson, Charles, and Veihmeyer (10) discuss the origin and synonymy of the fungus, stating that De Seynes found it on pineapple and described it under the name *Sporoschasma paradoxum*. Saccardo in 1892 gave it the genus name *Chalara*, the binomial becoming *Chalara paradoxa* (De Seynes) Sacc. The next year, Went in Java described a serious fungus disease of pineapple and designated the pathogene by the new generic name *Thielaviopsis* because it produced hyaline conidia endogenously in a manner similar to that of the genus *Thelavia* Zopf, and by the specific name *ethacetucus* because of the production of a pleasant, strong odor resembling ethyl acetate. Von Hohnel (5) observed that the fungus of De Seynes and that of Went were identical and established the priority of the specific name of the former, calling the fungus *Thielaviopsis paradoxa*, hence, *Thielaviopsis paradoxa* (De Seynes) Von Hohnel.<sup>7</sup> Butler (2) states that he found also a pycnidial stage of the fungus which might place it in the genus *Sphaconema*. The pycnidia were globose, hairy, and ostiolate, the ostiole being at the tip of a long bristlelike neck. The pycnidiospores were small (10 to 12 $\mu$  by 3 $\mu$ ), hyaline, unicellular. Patterson et al (10) mention the appearance of pycnidia in their cultures of the fungus, but do not describe this stage. Thus far the writers have observed no pycnidial stage on specimens or on the various laboratory media.

The literature in general describes the fungus as having creeping, almost hyaline hyphae which bear two spore forms: Microconidia, which are small (10 to 15 $\mu$  by 3.5 to 5 $\mu$ ), cylindrical, hyaline, and formed uniseriately within a hyphalike conidial case, and macroconidia (16 to 19 $\mu$  by 10 to 12 $\mu$ ), which are extruded in chains from the tips of short lateral hyphae, and which are brown, thick-walled, and ovate. Grown on glucose-potato agar at 27° C., a culture 2 weeks old had conidia of the following dimensions. The so-called macroconidia with thick walls, length 11 to 17 $\mu$ , width 7 to 15 $\mu$ , brown conidia that were extruded from conidiophores of the same morphology as those that bear the hyaline so-called microconidia, length 6 to 23 $\mu$ , width 4 to 8.5 $\mu$ , and hyaline microconidia, length 5 to 15 $\mu$ , width 3 to 7 $\mu$ . The contents of both kinds of conidia vary greatly and may be very guttulate and granular to perfectly homogeneous. As shown in Plate 1, *a*, the typical conidiophores bearing the microconidia are much elongated and swollen at the base, while those bearing the macroconidia (pl. 1, *b*) are approximately half as long as the first and of uniform diameter. However, these distinctions as to spores and conidiophores are very artificial, as one finds all gradations in size, color, and shape between the extremes described, and all the conidia are probably produced endogenously, although some are pictured which appear to originate

<sup>7</sup>Dade (2a) has found a fungus of the genus *Ceratostomella* which he considers to be the perfect stage of *Thielaviopsis paradoxa*, the name of the organism would thus become *Ceratostomella paradoxa*.

acrogenously In germinating on glucose-potato agar, the protoplast of the mature macroconidium bursts through a longitudinal slit and forms a globule of naked protoplasm which proceeds to grow into mycelium. (Pl. 1, *c*) In water, the conidia germinate directly by sending out a germ tube. Apparently the brown conidia need a rest period before they will germinate. The hyaline conidium germinates readily without a rest period, sending out one (pl 1, *d*), occasionally two, germ tubes from any place on its periphery. The hyphae are subhyaline with cross walls and show a strong tendency to anastomose and to form branches at right angles to the parent hypha. (Pl. 1, *e*)

#### PATHOLOGICAL HISTOLOGY

The cells of affected tissues turn brown as both walls and lumen become filled with gum. The formation of gum in palm fronds is not peculiar to this disease alone, for any wound induces a tendency to the formation of pentosanlike substances. In some sections the hyphae appear completely to fill some of the tracheae and parenchymatous cells. (Pl 2) These hyphae and the gum that forms in the pathological tissues may become so abundant as to interfere seriously with the transpiration stream and produce a permanent wilting of the pinnae several feet beyond the region invaded by the fungus. In addition to the intracellular growth, the fungus is found abundantly in the intercellular spaces but does not appear to grow in the region of the middle lamellae. Abundant fruiting occurs on the surface of a lesion and, as the decay progresses, within the disintegrated tissues.

#### CONTROL

In the West Indies, dipping in 4-5-40 Burgundy or Bordeaux mixture gave adequate control of the disease on sugarcane sets. In Jamaica, where the malady occurs on coconuts, the diseased tissues are excised and the wounds dressed with a mixture of equal parts of copper sulphate, salt, and lime. Patterson et al. (10) found formaldehyde gas (1,200 cubic centimeters commercial formalin per 1,000 cubic feet) effective in controlling the fungus on pineapples, even when the organism was inserted to a depth of half an inch. Simmonds<sup>8</sup> in Australia has utilized both benzoic acid and boric acid effectively in controlling decay of pineapples. In this work the copper fungicides were less effective than the two organic acids.

In the case of date palms it seems advisable to prune out the affected fronds, leaf bases, and inflorescences, and to protect the pruning cuts and surrounding tissues with some disinfectant. Some preliminary laboratory experiments made by the writers indicate that copper sprays and dusts may be effective. Bordeaux dust, a 5-5-50 Bordeaux mixture, and ammoniacal copper carbonate inhibited germination of the conidia in a weak glucose-potato broth or in 10 per cent sucrose solution, the last-named fungicide being slightly less effective than the Bordeaux. Calcium monosulphide dust, dry lime sulphur, liquid lime sulphur, 1 per cent boric acid, 1 per cent benzoic acid, and 1 per cent formalin were likewise effective in inhibiting germination. All the chemicals in liquid form except

<sup>8</sup> Verbal communication

the formalin were atomized onto glass slides and allowed to dry before the spore suspension was applied with an atomizer. Flowers of sulphur dust under the conditions of the experiment was entirely ineffective in preventing germination.

### SUMMARY

A fungous disease of economic importance has been found on date palms in California, Arizona, and northern Africa. A preliminary survey indicates that all varieties of the date palm are probably susceptible. The disease has been found occurring naturally on all parts of the plant except the roots and stem, and these latter organs have by artificial inoculation been found to be readily susceptible.

Typical lesions are dark brown to black, hard, carbonaceous, and in mass give the petioles, midrib, fruit strands, and fruit stalks a scorched appearance, which suggests "black scorch" as the common name. Many of the fruit strands may be completely severed by the attack and the crop materially lessened. Wounding was shown to be unnecessary for infection of the root, fruit strands, petiole, and pinnae. The decay is most serious when it attacks the terminal bud, either killing the palm, or, when not fatal, producing the so-called "fool disease" effect, in which the injured terminal bud grows out laterally, setting the normal growth of the palm back several years.

Both the hyaline and the brown spores of the fungus *Thielaviopsis paradoxa* (De Seynes) Von Hohnel are found on the surface of the lesions. The conidia originate endogenously in uniseriate chains from subhyaline conidiophores. The optimum temperature for the fungus in culture lies between 24° and 27½° C, it makes very little growth at 32°. The brown spores apparently need a rest period before germination. The hyaline conidium germinates readily without a rest period, sending out one, and occasionally two, germ tubes from any place on its periphery. In germinating on glucose-potato agar the protoplast of the mature macroconidium bursts through a longitudinal slit, liberating a globule of naked protoplasm which proceeds to grow into mycelium. The hyphae are subhyaline with cross walls and show a strong tendency to anastomose and to form branches at right angles to the parent hyphae.

A histological study of the petiole of a diseased frond showed the fungus growing intracellularly in tracheae and parenchyma and intercellularly in the intercellular spaces but not in the middle lamellae.

To control the malady, the affected fronds, leaf bases, and inflorescences should be pruned out and the pruning cuts and surrounding tissues protected with some disinfectant. Preliminary experiments indicate that copper sprays, dusts, and various other chemicals may be effective.

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# THE GLOSSY CHARACTER ( $gl_3$ ) IN MAIZE AND ITS LINKAGE RELATIONS<sup>1</sup>

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## INTRODUCTION

Glossiness in maize seedlings is inherited as a simple Mendelian recessive. At least three genetically different types of glossiness have been discovered, each dependent upon the homozygous recessive condition of a single factor. Glossy seedlings are characterized by a shiny, smooth, waxy appearance. A normal maize seedling has a slight silvery sheen on the surface which a glossy one lacks. When glossy seedlings are sprinkled, the water collects in drops on the surface of the leaves, while in normal seedlings the leaves shed the water completely. Classification of the two types in a segregating generation is therefore facilitated by sprinkling the leaves with water. Glossiness is apparent as soon as the leaf emerges from the soil, and may be observed until the plant is about 20 to 30 inches tall, when it begins to fade. By the time inflorescences are formed this character can no longer be differentiated.

The purpose of this study was to determine the normal mode of inheritance of glossiness ( $gl_3$ ) in seedlings and its linkage relations, and also to investigate the physical properties of the character and its effect upon the vigor of plants possessing it.

## REVIEW OF LITERATURE

During the last 20 years knowledge of linkage relationships in maize has developed to a point where the completeness of the chromosome map is second only to that of *Drosophila*. The comparative ease of culture, wide range of adaptation, type of inflorescence, large number of seeds per ear, and numerous observable genetic characters, all combine to make maize a good subject for genetic studies.

Beginning in 1929, Emerson and his coworkers at Cornell have each year summarized all the available linkage data in maize. The 1930 compendium lists approximately 100 genes combined into 10 linkage groups.<sup>3</sup> Populations for each cross are large, ranging from 500 to as many as 300,000 plants. Several 3-point relationships are given.

In 1921 Brunson discovered the strain now known as  $gl_1$ , in a first-year self progeny of a yellow dent corn from Illinois. A report of this discovery has never been published. In 1924 Kvakan (8)<sup>4</sup> reported a linkage relation of glossy seedling and brown aleurone. This was the first reference to the glossy character in the literature.

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<sup>2</sup> The writer wishes to express his thanks to Dr. H. K. Hayes, Chief of the Division of Agronomy and Plant Genetics, University of Minnesota, for his help in the conduct of this investigation.

<sup>3</sup> This material was as yet unpublished when the present paper was submitted.

<sup>4</sup> Reference is made by number (*italic*) to Literature Cited, p. 173.

The strain of glossy used was the simple Mendelian recessive discovered by Brunson, which has since been named  $gl_1$ . The linkage relations of glossy seedling ( $gl_1$ ) were summarized by Brunson in 1926 (2).

Hayes and Brewbaker (4) studied glossy seedlings in maize and made crosses between various strains. They described the appearance of glossy seedlings and reported two new ones phenotypically indistinguishable but genotypically distinct from the one obtained by Brunson. The 3 strains were designated  $gl_1$  (Brunson's),  $gl_2$ , and  $gl_3$ . Like  $gl_1$ , the new strains were each simple Mendelian recessives. It was stated that glossy seedlings appear rather frequently in selfed strains from various varieties. The results from crosses of 14 unknown glossies were given, each of these proving to be some one of the 3 reported.

In addition to establishing the existence of the two new glossy strains, Hayes and Brewbaker (5) found  $gl_2$  to be linked with  $F1$  (flinty endosperm). Finally, in 1930 Brewbaker and Hayes (1) published extensive data on the  $B-lg$  group, concluding that the order of the genes appeared to be  $(v_4-F1-ts_1)^{-}sk-gl_2-lg$ .

Except for a brief description of the glossy character by Hayes and Brewbaker (4), no investigation of the anatomy of glossiness appears to have been reported.

#### APPLICATION OF ORGANIC SOLVENTS TO THE LEAF SURFACE AS A MEANS OF ASCERTAINING THE ANATOMICAL NATURE OF THE GLOSSY CHARACTER

As was stated earlier, sprinkling with water helps in differentiating the two types of seedlings. Water will collect in drops on a glossy leaf but is shed by the leaves of normal seedlings. This distinction may be due to a difference in interfacial tension between the two systems concerned, that is, water-glossy and water-normal interfaces. If a normal leaf is held in a level position a drop of water may be balanced upon it. However, the drop will be almost spherical and with the slightest tipping of the leaf will roll rapidly in one direction or the other. On a glossy leaf a drop of water of the same size will be flat and will generally adhere to the leaf even when the surface plane is in a vertical position. Sometimes the water will spread out to form a rather thin film over the leaf surface, though at ordinary room temperature it usually remains in drops.

If a normal leaf is sprinkled with ethyl alcohol the results are very similar to those produced by an application of water to a glossy leaf. Drops of alcohol about the same size and appearance as drops of water on glossy leaves form on the normal surface. At 20° C, or about room temperature, the surface tension of water is 72.8 dynes per cubic centimeter while that of ethyl alcohol under the same conditions is only 21.7 dynes per cubic centimeter. This fact indicates that the difference in the ability of the two seedling types to collect water drops is due to interfacial tension.

Further observations are compatible with those just mentioned. Alcohol applied to glossy leaves quickly spreads out into a thin film. Salt water or ice water, both of which are a little higher in surface tension than pure water at room temperature, are readily repelled by normal seedlings. What appears to be a similar increase in interfacial tension can be seen when ice or salt water is placed on glossy

leaves, though drops will still be formed. Since normal leaves are so completely free from water and the drops on glossy ones are fairly frequent, a bed of segregating plants sprayed with salt solution or ice water can be classified a little more readily than when the usual method is used.

Glossy seedlings are slightly more transparent than normal ones. This fact can best be observed by examining plants that are deficient in chlorophyll but segregating for glossiness. When a glossy and a normal leaf from white or very light colored virescent seedlings are held up to the light, the glossy leaf will appear more transparent. If the leaves are soaked in chloroform for 5 to 10 minutes this difference will disappear and the normal will transmit as much light as the glossy. Furthermore, if a normal green seedling is washed thoroughly with chloroform or immersed in it for about five minutes it will become almost indistinguishable from a glossy plant. After this treatment the normal seedlings will collect water drops just as do the true glossy ones. The same effect may be produced with ether, but not with alcohol. It should be mentioned that after the chloroform washing, although normal leaves will collect water readily, the shape of the drops suggests that the interfacial tension is slightly higher than on real glossy leaves. However, this point requires further investigation with more refined technic.

Figure 1 shows a glossy seedling and a normal seedling on which one leaf was treated with chloroform as described above.

It is significant that dried leaves may also be classified into normal and glossy by means of the water test. If the shape of the epidermal cells had any great influence on the glossy properties it would seem that drying the leaves should destroy the difference. Furthermore, the treatment of dried normal leaves with chloroform will make them appear like glossy ones, as in the case of fresh leaves.

#### COMPARISON OF YIELDS FROM GLOSSY AND NORMAL PLANTS

A measure of the possible reduction in vigor, if any, associated with the glossy condition was obtained in the following manner. A segregating generation of the corn was grown in rows 3 feet apart with single plants in each hill and the hills 1 foot apart. One hundred and twenty-seven pairs of glossy and normal plants growing side by side were selected, and when mature, the ears of each plant were harvested individually. Care was taken to avoid having vacant hills either between the normal and glossy or in the nearest space on either side of the pair. The corn was then dried to a uniform moisture basis and the weight taken. In 61 of the pairs the normal plant was the more productive; in the other 66 pairs the glossy plant was the more productive. The average yield of normal plants was 102.09 gm. and of the glossy plants 100.44, a difference of  $1.65 \pm 4.32$  gm. In harmony with common observation, these results indicate that the presence of the glossy character detracts little, if any, from the general vigor of the plant. This vigor of homozygous glossy strains is one of the things that make the character particularly desirable for inheritance studies. These results indicate that the glossy character may be valuable as a marker in inbred lines or in crosses of corn used for commercial production, so that off pollinations may be readily detected and rogued out.

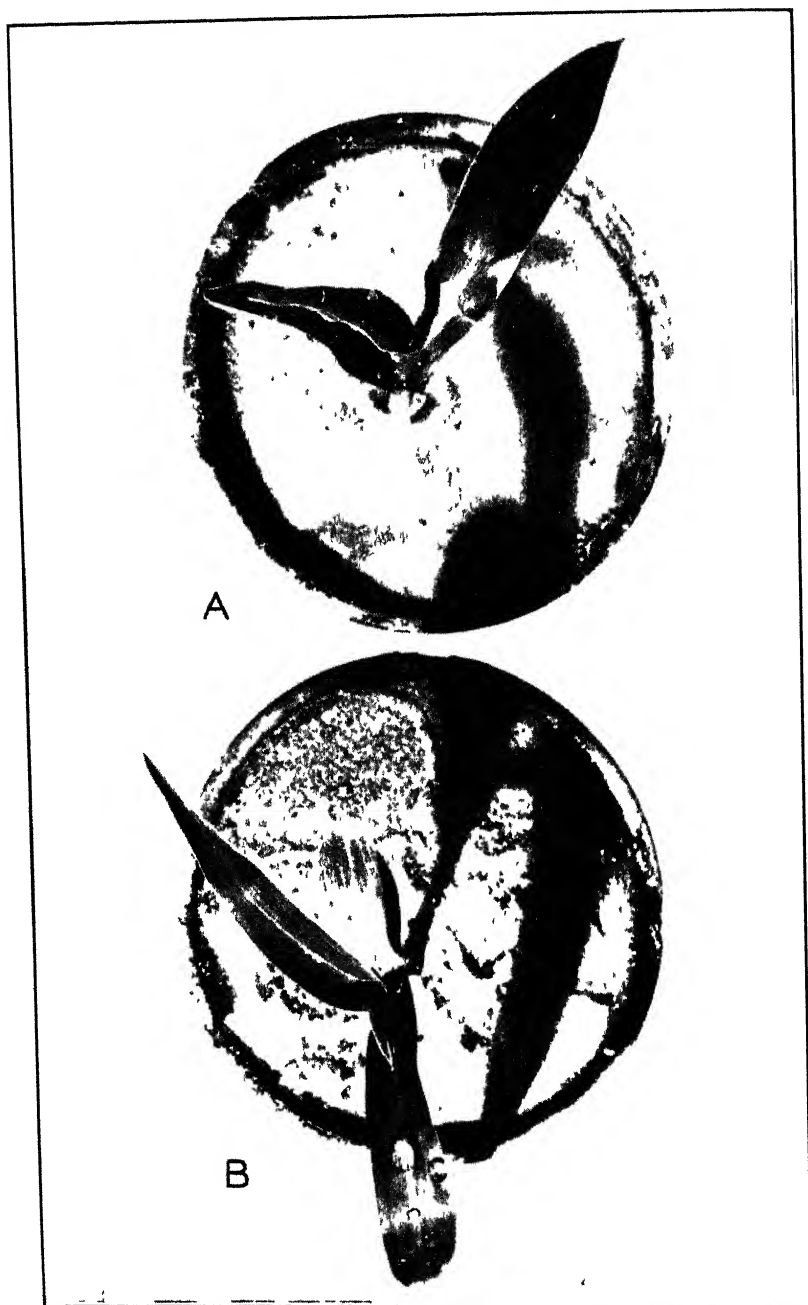


FIGURE 1—A, Typical glossy maize seedling that has been sprinkled with water. Note the water adhering to the surface. B, Normal maize seedling. The lowermost leaf has been washed with chloroform and the whole plant subsequently sprinkled with water. Several drops of water may be seen on the one leaf that is a "manufactured" glossy.

## INHERITANCE STUDIES

## METHODS

With a view to finding its linkage relation, a pure  $gl_3$  culture was crossed with as many chromosome "markers" as were available. The work was started in 1927. The  $F_1$  progenies from these crosses were back crossed to the double recessive in each case when it was practicable to do so. Some difficulty was experienced in getting double recessives. Where back crosses were not made the  $F_1$  progenies were selfed to produce an  $F_2$  population, and segregation of glossy in relation to the other character was studied by that means.

Since the glossy character may be differentiated in the seedling stage, a cross involving any other seedling difference or an endosperm character may be grown in the greenhouse and counts made there of segregating generations. This fact was taken advantage of in studying the crosses of  $gl_3$  with  $lg$ ,  $sh$ ,  $wx$ ,  $fl$ , and the four aleurone color factors  $A$ ,  $C$ ,  $R$ , and  $Pr$ .<sup>5</sup>

The recombination values from the back crosses were calculated by dividing the number of crossover gametes by the total number, since the constitution of the gametes produced by an  $F_1$  progeny can be read directly in a back cross. In the case of the  $F_2$  populations, recombination percentages and their probable errors were calculated from tables compiled by Immer (6), which are based on the work of Fisher and Balmukand (3).

TABLE 1.—*Linkage relations of  $gl_3$  with the  $su$ - $Tu$  group in maize*

Culture No	Year	Geno	Linkage phase	Number of individuals *				Total population	Recombination percentage
				AB	Ab	aB	ab		
H76 .....	1928	<i>su</i> .....	Repulsion $F_2$ .....	2,078	653	924	43	3,698	25.1±0.48
H200×H210 .....	1929	<i>su</i> .....	Repulsion, back cross .....	78	255	271	82	686	23.3±1.08
H260×H9 .....	1930	<i>su</i> .....	Repulsion .....	148	576	614	169	1,507	26.6±0.77
H26×H77 .....	1928	<i>Tu</i> .....	do. ....	1,093	172	107	1,195	2,597	10.88±0.41

\*  $Aa$  stands for  $Gl_3 gl_3$ ,  $Bb$  represents the other factor pair

LINKAGE RELATIONS OF  $Gl_3$  WITH THE  $su$ - $tu$  GROUP

Table 1 gives the results of two back crosses and of one  $F_2$  population which show that  $gl_3$  is linked with  $su$ . The  $F_2$  generation, consisting of 3,698 individuals, gave a recombination percentage of  $25.1 \pm 0.48$ . In one back cross, which numbered 686 plants, there was a recombination value of  $23.3 \pm 1.08$  and in the other back cross, which contained 1,507 plants, there was a recombination value of  $26.6 \pm 0.77$ . The variations in these percentages (23.3, 25.1, and 26.6) do not appear significant in the light of their probable errors.

One back cross involving  $gl_3$  and  $Tu$  was obtained. The result here was a recombination percentage of  $10.88 \pm 0.41$ .

According to the most recent summary of Emerson and his co-workers, data published by Jones and Gallastegui in 1919, and later and more extensive data from Eyster and from Emerson, give an average

<sup>5</sup> The genetic symbols used in the text are as follows:  $gl_1$ , glossy seedling,  $gl_2$ , glossy seedling,  $gl_3$ , glossy seedling,  $sh$ , shrunken endosperm,  $wx$ , waxy endosperm,  $fl$ , floury endosperm,  $Fl$ , flinty endosperm,  $su$ , sugary endosperm,  $Y$ , yellow endosperm;  $Tu$ , tunicate ear,  $ts$ , tassel seed,  $br$ , brachytic,  $v_2$ , virescent seedling,  $lg$ , liguleless leaf,  $g$ , golden plant,  $A$ ,  $C$ ,  $R$ , and  $Pr$ , aleurone color factors,  $B$ , intensifier of plant color

combination value of 28.6 per cent for *su* and *Tu*. Therefore the location of the genes is *su-gl<sub>3</sub>-Tu*. The mean of the three crosses here reported involving *su* gave a distance of 25 units from *su* to *gl<sub>3</sub>*. The distance from *gl<sub>3</sub>* to *Tu* (10.88) added to this gives a total of approximately 36, as compared to 28.6 for the distance from *su* to *Tu*. The distance, then, from *su* to *Tu* as previously reported is 7.4 units less than the sum of the distances *su* to *gl<sub>3</sub>* and *gl<sub>3</sub>* to *Tu* according to the data here presented. The probable error of the difference, 7.4 units, would be about 1 unit or slightly more since the probable errors of the average values concerned are all of approximately that size. In other words, this difference, though not very large, is statistically significant.



FIGURE 2—Seedlings from the segregating back-cross generation of the cross H260×H9 made in 1930, photographed in the greenhouse

This is as it should be if the amount of double crossing over between two genes is directly proportional to their distance apart (?).

Figure 2 shows the seedlings from the segregating back-cross generation of the cross H260×H9 made in 1930. (Table 1.) This cross was made in the repulsion phase, that is, *gl<sub>3</sub> gl<sub>3</sub> Su Su* × *Gl<sub>3</sub> Gl<sub>3</sub> su su*. The seedlings shown in the lower right half of the illustration are from starchy seeds, while those in the upper half are from sugary ones. The seedlings were sprinkled with water just before the picture was taken and the glossy ones may be distinguished by the drops of water on them. It will be noted that there are many more glossy seedlings in the starchy group.

TABLE 2—*Recombination values for  $Gl_3 gl_3$  with factor pairs in known linkage groups in maize*  
[Linkage phase, repulsion F<sub>1</sub>]

Culture No	Year	Gene	Linkage group	Number of individuals *				Total population	Recombination percentage
				AB	Ab	aB	ab		
II75.....	1928	<i>sh</i> .....	<i>C-wx</i> .....	2,703	818	810	304	4,635	53 0±0 71
II199.....	1929	<i>sh</i> .....	<i>C-wx</i> .....	2,572	711	801	238	4,322	51 0± 76
II73.....	1928	<i>wx</i> .....	<i>C-wx</i> .....	1,964	661	722	222	3,569	48 7± 86
II75.....	1928	<i>R</i> .....	<i>R-g<sub>1</sub></i> .....	2,580	941	808	306	4,635	50 5± 73
II72.....	1928	<i>R</i> .....	<i>R-g<sub>1</sub></i> .....	1,049	844	322	281	2,496	51 1±1 00
II76.....	1928	<i>R</i> .....	<i>R-g<sub>1</sub></i> .....	1,642	897	676	291	3,506	46 6± 88
II79.....	1928	<i>ts<sub>1</sub></i> .....	<i>B-lg</i> .....	1,199	361	314	79	1,953	47 5±1 17
II78.....	1928	<i>lg</i> .....	<i>B-lg</i> .....	677	264	265	80	1,286	46 4±1 46
II71.....	1928	<i>Fl</i> .....	<i>B-lg</i> .....	1,239	1,177	374	399	3,189	51 6± 88
II71.....	1928	<i>Y</i> .....	<i>Y-Pl</i> .....	1,793	623	597	176	3,189	47 7± 92
II82.....	1928	<i>br</i> .....	<i>P-br</i> .....	1,049	245	293	67	1,654	49 6±1 25
II72.....	1928	<i>Pr</i> .....	<i>Pr-v<sub>2</sub></i> .....	649	400	202	120	1,371	49 5±1 37
II76.....	1928	<i>Pr</i> .....	<i>Pr-v<sub>2</sub></i> .....	1,285	357	483	193	2,318	55 1± 99
II199.....	1929	<i>Pr</i> .....	<i>Pr-v<sub>2</sub></i> .....	2,420	863	770	269	4,322	49 7± 77

\* *Aa* stands for  $Gl_1 gl_1$ , *Bb* stands for other factor pair concernedPROOF OF INDEPENDENCE OF  $gl_3$  FROM SEVERAL LINKAGE GROUPS

In Table 2 data are presented from 16 F<sub>2</sub> populations, ranging from about 1,300 to 4,600 plants. Each of these populations gave a recombination percentage not deviating significantly from 50 per cent. The genes employed are *sh*, *wx*, *R*, *ts<sub>1</sub>*, *lg*, *Fl*, *Y*, *br*, and *Pr*. These data indicate that  $gl_3$  is not carried in chromosome groups *C-wx*, *R-g<sub>1</sub>*, *B-lg*, *Y-Pl*, *P-br*, or *Pr-v<sub>2</sub>*.

## SUMMARY

The homozygous glossy condition ( $gl_3$ ) has little, if any, effect on the general vigor of a corn plant. The true difference between normal and glossy seedlings appears to lie in the leaf cuticle. Exactly what this difference is has not been determined.

Inheritance studies have placed  $gl_3$  in the *su-Tu* linkage group. The order of the genes is *su-gl<sub>3</sub>-Tu*.

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# EFFECT OF THE HYDROGEN-ION CONCENTRATION OF THE SOIL ON THE GROWTH OF THE BEAN AND ITS SUSCEPTIBILITY TO DRY ROOT ROT<sup>1</sup>

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## INTRODUCTION

A more thorough knowledge of the environmental factors that affect the common bean (*Phaseolus vulgaris* L.) infected with the root parasite *Fusarium martii phaseoli* Burk. should be of considerable economic value. In certain sections of western New York the root rot caused by this organism is enphytotic, but the severity of the disease varies greatly, not only in different years but in different fields during the same year. In one field the lower roots of infected plants may be entirely rotted, leaving the plants supplied only by the surface roots; while in a neighboring field only the cortex of the tap-root and the finer rootlets will be invaded. In the latter case the reduction in yield is scarcely noticeable.

Certain environmental factors have been studied which might alter the severity of the disease and thus explain conditions such as those just described. It is the belief of Reddick<sup>2</sup> and of the writer<sup>3</sup> that while the soil temperatures found in the bean-growing sections of New York State may greatly influence the growth of the bean plant, they do not materially affect the course of the root-rot disease. Soil moisture, on the other hand, although a factor of some importance, has been found by the writer<sup>4</sup> to act only in a mechanical manner. If there is sufficient moisture in the soil, the roots, even though severely rotted, are able to supply water and inorganic food to the plant in such quantities that the reduction in yield of seed is slight. If the soil is dry, however, the yield may be reduced as much as 50 per cent. A third factor studied by the writer<sup>5</sup> directly concerns the pathogene. When *Fusarium martii phaseoli* is grown in pure culture its virulence is considerably reduced although such an attenuated culture may be restored to normal by growing it for several months on its host, the bean plant. It seems probable, although it has never been demonstrated, that a similar condition takes place in nature. After the fungus has grown saprophytically in the soil for a number of years it may cause only a light infection of the bean roots, but continued association with its host may build up its virulence.

The factors of soil moisture and the attenuation of the pathogene do not appear, however, to explain adequately all variations in the disease which are observed in the field. Other environmental factors

<sup>1</sup> Received for publication June 17, 1931, issued March, 1932.

<sup>2</sup> REDDICK, D. EFFECT OF SOIL TEMPERATURE ON THE GROWTH OF BEAN PLANTS AND ON THEIR SUSCEPTIBILITY TO A ROOT PARASITE. Amer Jour Bot 4 513-519 1917

<sup>3</sup> BURKHOLDER, W. H. THE EFFECT OF TWO SOIL TEMPERATURES ON THE YIELD AND WATER RELATIONS OF HEALTHY AND DISEASED BEAN PLANTS. Ecology 1 113-123, illus 1920

<sup>4</sup> ——— THE EFFECT OF VARYING SOIL MOISTURES ON HEALTHY BEAN PLANTS AND ON THOSE INFECTED BY A ROOT PARASITE. Ecology 5, 179-187 1924

<sup>5</sup> ——— VARIATIONS IN A MEMBER OF THE GENUS FUSARIUM GROWN IN CULTURE FOR A PERIOD OF FIVE YEARS. Amer Jour Bot 12 245-253 1925

that might affect its development were therefore sought. The hydrogen-ion concentration of the soil is known to alter the course of certain plant diseases, and a few preliminary observations indicated that it might affect the development of the dry root rot of the bean. Accordingly, an investigation was undertaken to determine the influence of this factor on (1) the growth of the plant and (2) on its susceptibility to attack by the dry root-rot organism.

It has been the general feeling among growers that an alkaline soil is necessary for a good production of beans, and this idea has crept into certain textbooks on vegetable gardening and field crops. Scattered references to beans in the reports of the extensive, but by no means recent, experiments conducted at the Rhode Island Agricultural Experiment Station on the effect of soil acidity and liming on plant growth indicate that the bean readily tolerates acid soils.

The present investigation was divided into two parts: (1) Experiments conducted in the greenhouse, in which many of the external factors were controlled, and (2) field surveys in the bean growing section of New York State.

#### GREENHOUSE EXPERIMENTS

Two series of controlled experiments were conducted, one during the early months of 1927 and the other in 1928. The experiments followed in outline earlier tests conducted by the writer<sup>6</sup> on the relation of soil moisture to this disease. Glazed jars of 1-gallon capacity filled with a rich garden loam of known hydrogen-ion concentration and water-holding capacity were used. The pH of the soil at the beginning of both the experiments was approximately 5.10. The hydrogen-ion concentration was determined electrometrically, a quinhydrone electrode being used. The water-holding capacity of the soil was determined by the Hilgard 1-cm. cup method.

In the first set of experiments, 50 culture vessels divided into 10 lots of 5 each were used. One lot was left untreated, and the hydrogen-ion concentration of each of the remaining lots was adjusted by the addition of sulphuric acid or sodium hydroxide. In 4 lots the hydrogen-ion concentration was increased by the addition of sulphuric acid. To each vessel in lot 1 was added 30 c. c. of a normal solution of sulphuric acid, and to each jar in lots 2, 3, and 4 were added, respectively, 70, 125, and 210 c. c. In 5 lots of vessels the hydrogen-ion concentration of the soil was lowered by the addition of a normal solution of sodium hydroxide in the following quantities: To each vessel in lot 1, 26.25 c. c.; lot 2, 52 c. c.; lot 3, 87.5 c. c.; lot 4, 122.5 c. c.; and lot 5, 175 c. c. The treated jars were allowed to stand until the soil became friable, and the soil was then mixed thoroughly and softened. This was necessary in the case of jars receiving large amounts of sodium hydroxide because the surface soil in these vessels became baked on drying.

The seed used in the experiment was a pure line of Wells Red Kidney. At the time of planting (March 13) a culture of the bean nodule organism was introduced into each vessel. A water suspension of spores of a recently isolated strain of *Fusarium maritimum phaseoli* was poured about the seed in three vessels in each lot of five, and an equal quantity of sterilized water was added to the check vessels. The soil

<sup>6</sup> BURKHOLDER, W. H. Op. cit. (See footnote 4.)

moisture throughout the entire series was held as uniform as possible by the method used in previous experiments<sup>7</sup>. At the beginning of the experiment the moisture content was adjusted to 50 per cent of the water-holding capacity of the soil. When the seed germinated all seedlings but two were removed, and the soil moisture was lowered to 45 per cent of its water-holding capacity. This percentage was maintained until blossoming time, when it was further lowered to 35.

The data obtained in this set of experiments are shown in Table 1. Three check cultures had to be discarded because of chance infection. The cultures are arranged in the table according to the amount of acid or alkali that each group received, and this arrangement follows, more or less, the hydrogen-ion concentration of the soil. The pH determinations were made at planting and at harvest. The hydrogen-ion concentration, it will be noted, decreased steadily in all vessels except four, and the change in a few others was slight. This decrease was on both untreated soil and on soil treated with acid and with sodium hydroxide.

TABLE 1—The effect of various hydrogen-ion concentrations of the soil, determined at planting and at harvesting, on the yield of healthy bean plants and on those infected with *Fusarium maritimum* phascoli

Experiment No <sup>a</sup>	p H of the soil		Yield	Experiment No <sup>a</sup>	pH of the soil		Yield
	At plant- ing	At har- vesting			At plant- ing	At har- vesting	
			<i>Grams</i>				<i>Grams</i>
1C.....	6 71	7 27	5 18	25C.....	5 13	5 94	8 34
2C.....	6 75	7 19	4 48	26I.....	5 13	5 36	5 79
3I.....	6 67	6 75	2 22	27I.....	5 07	5 69	5 18
4I.....	6 73	6 75	91	28I.....	5 16	5 69	7 45
5I.....	6 69	6 85	1 69	29C.....	4 86	5 39	5 34
6C.....	6 34	6 75	5 12	30I.....	4 85	5 34	6 02
7I.....	6 30	6 68	2 97	31I.....	4 83	5 18	4 98
8I.....	6 34	6 72	6 00	32I.....	4 88	5 31	4 40
9I.....	6 28	6 70	4 97	33C.....	4 66	4 77	4 54
10C.....	5 79	6 32	5 46	34C.....	4 62	5 00	3 70
11C.....	5 92	6 73	5 87	35I.....	4 60	4 67	3 00
12I.....	5 78	6 36	5 21	36I.....	4 63	4 88	4 31
13I.....	6 07	6 71	5 26	37I.....	4 66	4 89	2 20
14I.....	5 95	6 53	6 24	38C.....	4 43	4 57	1 49
15C.....	5 62	6 56	8 72	39C.....	4 44	4 44	1 65
16I.....	5 66	6 05	4 54	40I.....	4 39	4 72	1 05
17I.....	5 54	6 16	5 91	41I.....	4 31	4 47	61
18I.....	5 63	6 14	5 09	42I.....	4 42	4 76	1 07
19C.....	5 43	6 30	7 28	43C.....	4 16	4 10	-----
20C.....	5 50	6 08	6 85	44C.....	4 09	-----	09
21I.....	5 49	5 40	5 37	45I.....	4 09	4 25	-----
22I.....	5 41	5 66	5 30	46I.....	4 11	4 04	-----
23I.....	5 47	5 93	6 16	47I.....	4 09	4 19	33
24C.....	5 12	5 60	7 53				

a When experiment numbers are followed by C, they refer to checks, when by I, they refer to inoculated plants

A possible objection to this set of experiments is that the hydrogen-ion concentration of the soil does not extend to the alkaline side of neutrality. Preliminary tests on adjusting small quantities of this soil with sodium hydroxide showed that it should reach a pH of 8. However, because of the large quantities of soil used and the method of handling, it was not possible to obtain pH values above 7. On the other hand, when the soil was adjusted with sulphuric acid the

<sup>7</sup> BURKHOLDER, W. H. Op. cit. (See footnote 4)

hydrogen-ion concentrations determined in the preliminary tests were reached.

A second experiment was conducted in the early months of 1928. This experiment differed from the first in that an acid soil was used and the hydrogen-ion concentration was adjusted with hydrated lime. Forty-five culture vessels were divided into nine lots of five each. Lot 1 remained untreated, but to each jar in lot 2, 4 gm. of lime was added and to each jar in the remaining lots the application was successively increased by 4 gm. The lime was thoroughly mixed with the soil and allowed to stand for eight days, the moisture content being held at 50 per cent of the water-holding capacity. At the end of the eighth day lime was still visible in the jars that received large applications. In order that an equilibrium might be hastened all the jars were steam sterilized. The result of this process was not entirely satisfactory, however, for the hydrogen-ion concentration of the soil in all the jars decreased appreciably, whether or not they had received an application of lime. Nevertheless, the experiment was continued since the series covered the alkaline side of neutrality, the side that had not been reached in the previous experiments. Except for the use of lime and the sterilization of the soil, these jars were handled like those in the first experiment. The data collected are presented in Table 2. Where some of the check cultures are missing it is due to the fact that the plants became infected and were discarded.

TABLE 2—The effect of various hydrogen-ion concentrations of the soil at time of planting, podding, and harvesting, on the yield of healthy bean plants and on those infected with *Fusarium maitii* phaseoli

Experiment No. <sup>a</sup>	pH of the soil			Yield	Experiment No. <sup>a</sup>	pH of the soil			Yield
	At plant- ing	At pod- ding	At har- vesting			At plant- ing	At pod- ding	At har- vesting	
				Grams					Grams
1C-----	8.43	7.76	8.27	1.57	22I-----	7.76	6.96	4.49	-----
2C-----	8.04	7.69	7.82	4.03	23I-----	7.66	7.34	7.74	6.12
3I-----	8.11	7.69	7.66	-----	24C-----	7.51	7.30	7.44	6.10
4I-----	7.72	7.72	7.79	.28	25I-----	7.40	7.08	7.27	4.74
5I-----	7.89	7.56	7.79	.95	26I-----	7.27	6.98	7.05	3.87
6C-----	8.09	7.69	8.23	4.72	27I-----	7.30	7.35	7.52	7.92
7C-----	8.03	7.77	8.11	4.90	28C-----	7.15	7.42	7.17	8.52
8I-----	8.01	7.79	8.25	4.85	29C-----	7.24	7.07	7.06	8.17
9I-----	8.03	7.79	8.28	4.37	30I-----	7.39	7.32	7.32	6.92
10I-----	7.84	7.29	7.61	.20	31I-----	6.73	-----	6.61	5.51
11C-----	7.83	7.62	8.01	7.05	32I-----	7.57	7.47	7.37	3.27
12I-----	7.86	7.59	8.30	6.15	33C-----	6.91	6.90	6.44	7.72
13I-----	7.91	7.44	7.97	2.90	34I-----	6.93	6.10	6.31	4.82
14I-----	7.89	7.89	7.86	5.97	35I-----	7.24	7.42	7.07	4.92
15C-----	7.47	6.71	7.66	5.70	36I-----	6.90	6.86	6.95	1.88
16C-----	8.21	8.08	8.35	7.55	37C-----	6.41	6.48	6.61	1.75
17I-----	7.78	7.07	7.59	5.15	38C-----	6.41	6.02	5.90	1.32
18I-----	7.90	7.49	7.94	3.01	39I-----	5.87	6.30	5.87	3.05
19I-----	7.92	7.52	7.74	4.75	40I-----	6.00	5.98	5.51	2.77
20C-----	8.08	7.89	8.05	7.67	41I-----	6.34	6.50	6.05	5.27
21I-----	7.44	7.30	7.54	6.98					

<sup>a</sup> When experiment numbers are followed by C, they refer to checks, when by I, they refer to inoculated plants

The data in Table 2 are grouped in a manner similar to those in Table 1; that is, according to the amount of lime that each jar received. This arrangement follows, only in a general way, the hydrogen-ion concentration of the soil. The soil reaction of jars under like treatment varied considerably for no apparent reason, and the

variation from planting time to harvest was not always consistent. While a uniform series of cultures would have been highly desirable it was not absolutely necessary, since the object of the experiment was to determine whether or not the hydrogen-ion concentration of the soil at any degree would have an inhibiting effect upon the disease.

In Tables 1 and 2 an attempt was made to use the yield of seed in each culture vessel as an index of the amount and severity of the root rot present. This attempt, however, was not successful. There appeared to be too many factors besides root rot that influenced the yield. Some of these were unknown and others were difficult to control. For example, in the second set of experiments the greenhouse in which the plants were growing was very warm for several days at blossoming time because of the brightness of the sun, and many blossoms dropped. The drop was uneven, and at harvest time it was noted that certain plants, in the check cultures especially, had endeavored to even up this inconsistency by the production of late blossoms. The pods from these blossoms did not produce seed, however, so no record of them appears in the data.

In spite of the variations in yield shown in Tables 1 and 2 it may be seen that there is a decided tendency for plants from inoculated seed to produce fewer seed than the check plants. Moreover, this decrease in yield of plants from inoculated seed does not vary in any direct relationship with the hydrogen-ion concentration, and this at least would indicate that soil reaction has no effect upon the root rot of the bean. Furthermore, disregarding the data on yields, the same conclusions were reached when the roots of the plants were examined at harvest time. All roots of inoculated plants appeared to be equally infected, scarcely any variation in the severity of infection being discernible. This last observation is of greater importance than the data on yield, and mainly because of this finding further experiments of this type were discontinued.

Before leaving these experiments, however, it should be pointed out that the data in Table 1 seem to indicate that the red kidney bean will grow well in a soil with a fairly high hydrogen-ion concentration. A decrease in yield of healthy plants occurred only after the pH of the soil had dropped below 5.

#### FIELD SURVEYS

Along with the two sets of greenhouse experiments a survey of bean fields in western New York was made during August in 1928 and 1929. The surveys were made each year at as nearly the same time as possible so that the data would be comparable if a seasonal variation in the hydrogen-ion concentration of the soil occurred. On these surveys the following procedure was employed: First, the variety of beans was determined and an estimate was made of the yield, then the degree of severity of root-rot infection was noted. If other diseases or factors were present that might contribute to a decrease in yield they, too, were recorded. A group of bean plants was then selected which appeared typical of the field at large and three samples of soil were taken about the bean roots within a foot or more of each other. While the hydrogen-ion concentration might vary over the field, it was felt that a determination should be made near a typical group of plants. The three soil samples were mixed, placed in a paper bag, and taken to the laboratory for pH determinations.

In 1928 the survey began in southern Monroe County and proceeded south through Genesee County into the white marrow section of Wyoming County. The high alkaline soils were found in the limestone area near Garbut and the acid soils about Perry and Castile. The data from this survey are shown in Table 3. In 1929 the survey was made in some of the newer bean-growing sections, whereas in 1928 the survey was made in a section where beans had been grown for many years. This probably accounts for the fact that more fields were found to be contaminated with *Fusarium maritii phaseoli* the first year than were found the second year. The data collected in 1929 are presented in Table 4.

TABLE 3—*Hydrogen-ion concentration of the soil of bean fields contaminated with or free from the root parasite Fusarium maritii phaseoli*

[Determinations made August, 1928]

Sample No	pH of soil	Variety grown	Extent of infection	Yield of beans
1	8.67	Pea	Severe	Poor
2	8.64	do.	None	Excellent
3	8.45	do.	Severe	Fair
4	8.30	do.	Light	Good
5	7.96	Medium	do.	Fair
6	7.75	Pea	Trace	Poor <sup>a</sup>
7	7.66	do.	None	Good
8	7.32	Red kidney	Light	Fair
9	7.03	Marrow	do.	Good
10	6.93	Blue pod medium	Moderate	Fair
11	6.88	White marrow	Severe	(?)
12	6.83	do.	Moderate	Fair
13	6.64	Red kidney	Trace	Good
14	6.58	White marrow	Very severe	Poor
15	6.31	do.	do.	Do
16	6.25	Red kidney	Light	Do <sup>b</sup>
17	6.05	White marrow	Moderate	(?)
18	6.02	Red kidney	Light	Good
19	5.95	White marrow	do.	Do
20	5.78	do.	do.	Fair
21	5.54	do.	Very severe	Poor
22	5.32	do.	Trace	Good
23	5.29	do.	Moderate	Do
24	5.27	do.	Severe	Fair
25	5.27	do.	Very severe	Poor
26	5.19	do.	do.	Do
27	5.02	do.	Trace	Do
28	5.02	Yellow eye	Light	Good
29	5.00	White marrow	Moderate	Poor
30	4.99	do.	Light	Good
31	4.99	do.	Trace	Fair
32	4.97	do.	None	Good
33	4.95	Yellow eye	Trace	Fair

<sup>a</sup> Anthracnose also present in the field which might have decreased yield.

<sup>b</sup> Anthracnose and bacterial blight also present and might have decreased yield.

TABLE 4.—*Hydrogen-ion concentration of the soil of bean fields contaminated with or free from the root parasite Fusarium martii phaseoli*

[Determinations made August, 1929]

Sample No	pH of soil	Variety grown	Extent of infection	Yield of beans
1.....	8.30	Pea.....	Very severe.....	None
2.....	8.19	do.....	Trace.....	Poor <sup>a</sup>
3.....	8.19	White marrow.....	Light.....	Good
4.....	8.18	Red kidney.....	Trace.....	Fair
5.....	8.08	White marrow.....	Severe.....	Poor
6.....	7.97	do.....	do.....	Do
7.....	7.81	Red kidney.....	None.....	Good
8.....	7.84	Pea.....	Trace.....	Do
9.....	7.79	Red kidney.....	None.....	Do
10.....	7.57	White marrow.....	Moderate.....	Fair
11.....	7.49	Red kidney.....	Trace.....	Good
12.....	7.47	Blue pods.....	do.....	Fair
13.....	7.40	White marrow.....	None.....	Good
14.....	7.35	Red kidney.....	Trace.....	Fair, <sup>b</sup>
15.....	7.34	White marrow.....	Severe.....	Poor, <sup>c</sup>
16.....	7.32	Red kidney.....	Trace.....	Good, <sup>d</sup>
17.....	7.29	do.....	None.....	Fair <sup>c</sup>
18.....	7.25	do.....	Light.....	Good
19.....	7.25	White marrow.....	do.....	Do
20.....	7.08	do.....	Very severe.....	Poor <sup>d</sup>
21.....	7.07	do.....	Trace.....	Do <sup>a</sup>
22.....	7.03	Pea.....	Severe.....	Do
23.....	7.03	White marrow.....	Trace.....	Good
24.....	6.98	Red kidney.....	Light.....	Do
25.....	6.91	White marrow.....	Moderate.....	Fair
26.....	6.91	Pea.....	None.....	Poor <sup>a</sup>
27.....	6.71	White marrow.....	Moderate.....	Good
28.....	6.70	do.....	None.....	Poor <sup>b</sup>
29.....	6.51	White imperial.....	do.....	Good
30.....	6.44	Red kidney.....	do.....	Fair <sup>a</sup>
31.....	6.39	do.....	do.....	Do
32.....	6.27	Pea.....	do.....	Good
33.....	6.02	White marrow.....	Moderate.....	Poor <sup>c</sup>
34.....	5.97	Pea.....	None.....	Fair
35.....	5.90	Red kidney.....	do.....	Good, <sup>d</sup>
36.....	5.80	Pea.....	do.....	Do.
37.....	5.70	do.....	do.....	Fair
38.....	5.68	do.....	Light.....	Do, <sup>e</sup>
39.....	5.62	Red kidney.....	do.....	Do.
40.....	5.54	White kidney.....	Trace.....	Do
41.....	5.48	Pea.....	Severe.....	Poor

<sup>a</sup> Dry weather may have decreased yield<sup>b</sup> Planted late, which may have decreased yield<sup>c</sup> Bacterial blight may have decreased yield<sup>d</sup> Insects may have decreased yield

Tables 3 and 4 show that severe infection may be produced by the root-rot organism in a soil of fairly high hydrogen-ion concentration (pH 5.0) and in an alkaline soil (pH 8.0) as well. This is in harmony with the data gathered in the greenhouse experiments. The tables show further that the range of hydrogen-ion concentration of the soil in the bean-growing section of New York is not a limiting factor in crop production. A good yield may be produced in both acid and alkaline soils, and this also agrees with the data in Tables 1 and 2. If certain varieties of beans are less acid tolerant than others it is not apparent from these surveys.

### CONCLUSIONS

The bean plant (*Phaseolus vulgaris*) appears to be little affected by the hydrogen-ion concentration of the soil, and thrives well in an acid or in an alkaline soil.

In the bean-growing section of New York State the susceptibility of the bean plant to *Fusarium martii phaseoli* is not affected by the hydrogen-ion concentration of the soil.



# RELATION OF TEMPERATURE TO ANTHESIS AND BLOSSOM DROP OF THE TOMATO, TOGETHER WITH A HISTOLOGICAL STUDY OF THE PISTILS<sup>1</sup>

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## INTRODUCTION

Under certain conditions and in practically all parts of the country large numbers of tomato blossoms fail to set fruit. This abortion and dropping of flowers may be so great as to reduce the crop materially. Abscission, which results in the dropping of the flowers, may occur before, during, or shortly after anthesis. This problem has received attention from investigators throughout the country. Usually, however, it has been considered from the standpoint of the nutrition of the plant and the influence of unfavorable weather conditions. It is not known whether these conditions affect the formation of the embryo sac, the fertilization process, or the development of the embryo and endosperm. This paper presents (1) the results of observations on the effect of temperature on anthesis and blossom drop and (2) the results of a histologic study of normally developing and dropping blossoms, with special reference to the development of the embryo sac, embryo, and endosperm.

## REVIEW OF LITERATURE

Thompson<sup>4</sup> has enumerated the following as possible causes of blossom drop: (1) A sudden appearance of cold or cool weather when the plants are in blossom; (2) hard rains which may wash away the pollen or otherwise affect pollination; (3) very hot, dry weather, especially drying winds; (4) injury by thrips; and (5) rapid vine growth resulting from an excess of nitrogen in the soil.

Investigations to determine the causes of the shedding of tomato blossoms have been carried on in Oklahoma for at least 14 years by Morris, Booth, Herron, Cross, and White.<sup>5</sup> These investigators have studied the following as probable causes: (1) Extremely hot weather, (2) overrapid growth due to an excess of nitrogen fertilizers, (3) lack of proper nutrients in the soil, (4) deleterious substances in the soil, (5) insect injury, (6) hot dry winds, (7) lack of moisture, (8) extremes of temperature, (9) varietal susceptibility, (10) poor pollination, and (11) low humidity. Morris conducted variety, pruning, and soil-fertility tests but obtained successful results of only minor importance. He also shaded some of the plants but apparently found no direct evidence of the cause of the trouble. Booth continued the varietal studies, and also inaugurated studies in the relation of humidity, irrigation, diseases, thrips, and pollination to fruit produc-

<sup>1</sup> Received for publication July 13, 1931, issued March, 1932.

<sup>2</sup> The author is indebted to Ephraim Huxson and John Faulkner for assistance in a part of this investigation.

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<sup>4</sup> THOMPSON, H. C. VEGETABLE CROPS. 478 pp., illus. New York, 1923.

<sup>5</sup> MORRIS, O. M., BOOTH, N. O., HERRON, L. C., CROSS, F. B., and WHITE, R. E. FAILURE OF TOMATO BLOSSOMS TO SET FRUIT. Okla. Agr. Expt. Sta. unpublished data.

tion. The number of fruits produced under conditions resulting from irrigation was increased 18 per cent. Herron concluded from the results of windbreak tests that hot dry winds have little effect on the dropping of the blossoms. Blossom drop was not reduced by shading the plants nor by pruning and training them. Heavy fertilization with sodium nitrate, potassium sulphate, and acidulated rock phosphate applied in separate plots likewise failed to reduce the amount of blossom drop. Thrips, *Euthrips tritici projectus*, were found in abundance, but no constant relationship was observed between the number of thrips present and the number of blossoms that dropped.

Radspinner<sup>6</sup> continued the work at Oklahoma for several years, and in 1922 summarized the data. He noticed that the dropping was most severe when soil moisture was deficient or when the temperature was high or the humidity low. Fertility of soil influenced but slightly the dropping of blossoms.

Cross, working at Oklahoma in 1924 and 1925, divided the fruiting season into three periods. He found that blossoms were shed at all times during the season, but that the percentage shed was low during the first and third periods and high during the second. By far the greater number of blossoms produced during July and August were shed. Cross also found that a large percentage of shedding blossoms had injured floral parts. This he attributed to the fact that hot dry winds withdraw moisture from the organs, causing them to blacken and die. This injury prevents normal pollination and fertilization. He concluded that the greater part of the blossom drop was caused by excessively hot dry winds and intense heat of the sun.

Kendall<sup>7</sup> thinks that physiological conditions within the plant, as influenced by soil moisture and fertility, are the chief causes of floral abscission in the field. Kraybill<sup>8</sup> showed that the number of blossoms that dropped was influenced by the amount of available mineral nutrients under conditions of uniformity with respect to other external factors.

## MATERIAL AND METHODS

All the tests were made at the Oklahoma Agricultural Experiment Station. The Bonny Best variety of tomato, grown during 1929 as a fall crop in the greenhouse and handled in a commercial manner, was used in these studies. All plants were pruned to one stem. Collections for the study of the development within the pistils after anthesis were made at certain intervals after emasculated blossoms had been hand-pollinated. Pollinations were made on the day of anthesis. Samples of naturally aborting flowers and emasculated, unpollinated flowers were collected at the same time that collections of developing flowers were made. The pistils were killed and fixed in Karpechenko's solution, dehydrated, embedded in paraffin, sectioned, and stained in the usual manner. Haidenhain's haematoxylin stain was used. For tracing pollen-tube growth the sections were also stained with resorcin blue after the second iron-alum solution in the schedule for the haematoxylin stain.

<sup>6</sup> RADSPINNER, W. A. EFFECTS OF CERTAIN PHYSIOLOGICAL FACTORS ON BLOSSOM DROP AND YIELD OF TOMATOES. Amer Soc Hort Sci Proc (1922) 19: 71-75. 1923

<sup>7</sup> KENDALL, J. N. ABSCISSION OF FLOWERS AND FRUITS IN THE SOLANACEÆ, WITH SPECIAL REFERENCE TO NICOTIANA. Calif Univ Pubs, Bot. 5 [347]-428, illus. 1918 (Thesis, Ph. D., Univ Calif., 1917)

<sup>8</sup> KRAYBILL, H. R. EFFECT OF NUTRITION ON THE NUMBER OF BLOSSOMS PER CLUSTER AND THE DROPPING OF BLOSSOMS IN THE TOMATO. Amer Soc Hort. Sci Proc (1925) 22: 371-374. 1926

During the spring and summer of 1930 information was obtained as to the time and amount of anthesis and the time and amount of abortion of these flowers on plants grown out of doors. Ten plants of equal size and age were selected for study. The number of flowers opening each day on the different plants from June 8 to July 17 and the number dropping each day from June 12 to July 16 were recorded.

TABLE 1 — *Tomato anthesis from June 8 to July 17, 1930*

Date	Number of flowers opening on plant										Total	Average
	1	2	3	4	5	6	7	8	9	10		
June 8	1	0	1	0	0	0	0	0	0	0	2	0.2
9	1	2	0	1	0	0	0	0	0	0	4	0.4
10	1	2	0	1	0	1	0	0	0	0	5	0.5
11	3	2	4	1	1	0	2	0	2	0	15	1.5
12	1	0	0	0	0	1	0	1	0	0	4	0.4
13	1	0	0	0	0	0	0	1	0	0	2	0.2
14	0	1	0	0	1	0	0	0	0	1	3	0.3
15	1	0	0	2	2	0	1	0	1	0	7	0.7
16	0	1	2	1	0	2	0	2	0	1	9	0.9
17	3	3	1	2	2	2	1	0	1	0	15	1.5
18	1	4	2	1	3	1	3	1	2	1	19	1.9
19	2	6	3	6	3	5	2	6	0	5	38	3.8
20	2	4	4	2	2	2	4	2	3	3	28	2.8
21	3	2	3	2	3	1	2	3	2	0	21	2.1
22	3	2	2	3	1	3	2	2	3	0	21	2.1
23	1	2	1	1	2	1	1	2	0	0	11	1.1
24	2	4	3	4	6	2	2	6	0	2	31	3.1
25	3	6	1	5	3	2	2	2	2	5	31	3.1
26	1	2	2	2	3	7	5	7	5	0	34	3.4
27	1	6	2	6	1	7	1	4	1	1	30	3.0
28	1	3	2	13	2	2	6	5	3	7	44	4.4
29	3	3	2	5	2	3	2	5	3	3	31	3.1
30	0	1	1	0	2	0	1	3	1	0	9	0.9
July 1	1	1	1	0	2	3	2	3	2	4	19	1.9
2	1	4	0	0	4	1	5	5	4	4	28	2.8
3	5	0	4	1	3	1	5	1	2	3	25	2.5
4	3	2	2	0	3	2	4	3	7	0	20	2.0
5	2	2	1	0	3	3	6	2	2	6	32	3.2
6	2	2	1	0	1	0	2	2	6	0	27	2.7
7	3	0	2	1	3	0	1	2	2	1	13	1.3
8	1	1	2	1	3	2	2	2	0	2	28	2.8
9	0	0	4	2	2	4	5	2	1	1	20	2.0
10	1	0	0	2	0	2	1	1	1	3	11	1.1
11	3	0	0	3	2	6	3	2	1	3	23	2.3
12	2	1	2	4	3	3	5	0	3	5	28	2.8
13	5	0	6	3	3	3	6	0	2	4	32	3.2
14	2	0	3	2	5	3	7	0	4	5	31	3.1
15	2	1	1	0	2	2	5	3	0	1	17	1.7
16	2	1	4	4	1	1	2	1	0	0	16	1.6
17	4	2	2	4	1	2	3	1	0	1	20	2.0
Total	73	73	70	90	84	82	108	82	65	72	799	-----

## OBSERVATIONS

## INFLUENCE OF TEMPERATURE ON ANTHESIS AND BLOSSOM DROP

Table 1 gives the number of flowers that opened each day on the different plants from June 8 to July 17. After July 17 the taking of records was discontinued. The data show that tomato plants have no one definite flowering peak. In most plants there was a large production of flowers from June 14 to 29, and this was followed by a series of production peaks until the records were discontinued. The coefficient of correlation of temperature and amount of flowering, when temperature and anthesis for the same day are considered, was  $-0.21$ . However, if temperature affects anthesis, some time must elapse between the time that the effect is exerted and the visible effect on flowering. For this reason the coefficients of correlation

of temperature 1, 2, 3, and 4 days before the day of anthesis and the amount of flowering were calculated. The coefficient of correlation of temperature 1 day before anthesis and the amount of flowering was  $-0.139$ ; of temperature 2 days before anthesis,  $0.113$ ; of temperature 3 days before anthesis,  $0.499$ ; but for 4 days before anthesis it dropped to  $0.435$ . It therefore appears that there is approximately a 3-day lag between the time that temperature affects anthesis and the time that the effects become visible.

The growing season of 1930 was extremely dry; less than 2 inches of rain fell during the growth of the plants, that is, up to the time the records were discontinued. Flowering may also be dependent upon

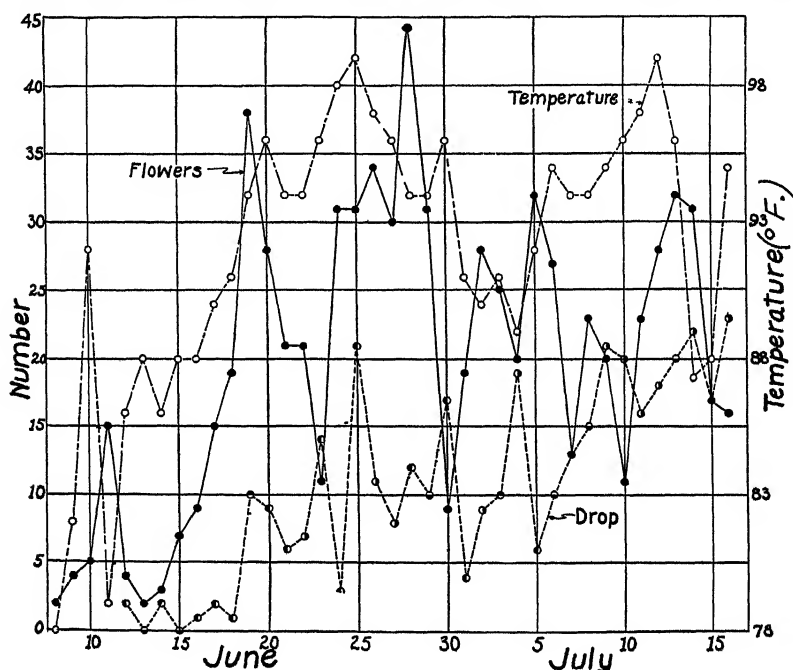


FIGURE 1—Flowering and flower-drop records for tomatoes during June and July, 1930, with mean temperature. Averages of all plants are shown.

rainfall or soil moisture as well as temperature. This may explain the lack of better correlation between temperature and anthesis.

Table 2 and Figure 1 give the number of flowers that dropped each day from the same 10 plants from June 12 to July 16, when the records were discontinued.

An analysis of the data in Figure 1 indicates that the relation of temperature to blossom drop is similar to the relation of temperature to anthesis. The coefficient of correlation of temperature and amount of blossom drop, when temperature and blossom drop for the same day are considered, was  $0.413$ . The value of  $r$  when temperature 1 day before blossom drop is considered was  $0.476$ ; for 2 days before the drop,  $0.499$ ; for 3 days before the drop,  $0.68$ ; but for 4 days before the drop the value of  $r$  decreased to  $0.63$ . As in the case of temperature and anthesis, these data indicate that there was a 3-day lag between the time that temperature exerted an effect on blossom drop and the time that the effect became visible.

TABLE 2—*Blossom-drop data on tomatoes from June 12 to July 16, 1930*

Date	Number of flowers dropped from plant No.										Total	Average
	1	2	3	4	5	6	7	8	9	10		
June 12	1	1									2	0.2
13			1								0	0
14		1	1								2	0.2
15											0	0
16			1								1	0.1
17	1	1									2	0.2
18	1										1	0.1
19	2	1		1	1	1		1			10	1.0
20	2	2	1		1		1				9	0.9
21	1	1		1	1	1	1				6	0.6
22		4		1	1	1					7	0.7
23	3	7	2		2						14	1.4
24	2		1								3	0.3
25		8	2	4	2		2	2		1	21	2.1
26	4	4	1	1	1						11	1.1
27	1	3	1	2				1			8	0.8
28		2	2	2		3	1	2			12	1.2
29		2	2	2			1	3		2	10	1.0
30	1	6		6	1	1	1		1		17	1.7
July 1		1		2				1			4	0.4
2	1		2	1		2				2	9	0.9
3		2	1		1	1	2	2		1	10	1.0
4	4	3	1		1		4	3	2	1	19	1.9
5	2						1		2	1	6	0.6
6	2	1	1			1	2	1			10	1.0
7			2	1	3	1	2		3	1	13	1.3
8	1	2	2	1	1	1	2	2	2	1	15	1.5
9			2	1	5	3	3	2	3	2	21	2.1
10	2	2	1	2	2	2	3	1	3	2	20	2.0
11			2	1	3	3	2	1	3	1	16	1.6
12	2	1	3	2	2	2	1	2	2	1	18	1.8
13	2	1	2	3	2	1		4	3	2	20	2.0
14	3	3	1	2	3			2	3	3	22	2.2
15	2	2	2	1	2	2	1	1	2	2	17	1.7
16	1	4	2	2	3		2	2	3	3	23	2.3
Total	41	65	36	39	38	27	34	34	34	31	379	-----

Observations indicate that the amount of blossom drop was greatly increased by low humidity, hot dry winds, and low soil moisture. This finding agrees with the results obtained by Cross at the Oklahoma station in 1924 and 1925. During the periods of high temperature and low humidity, practically all the styles elongated abnormally. In most instances this elongation occurred before the flowers opened and before the anthers dehisced. (Pl. 1, A) Thus the stigma and style were exposed to the desiccating winds and they soon became wilted and dried up. Practically 100 per cent of such flowers abort.

#### ABORTING AND DEVELOPING PISTILS

The external characteristics typically distinguishing the aborting from the developing flowers are a yellowing pedicel and pistil, shriveled blossom, and the development of the abscission layer. Aborting flowers drop naturally or readily fall when jarred or touched.

#### DEVELOPMENT OF EMBRYO AND ENDOSPERM IN NORMAL PISTILS

The ovules of the tomato are very numerous, completely covering the free surface of the placenta. (Pl. 1, B) The integument consists of a thick band of tissue closely surrounding the nucellus. It is often difficult to differentiate the integument from the nucellus. As the embryo sac enlarges the cells of the nucellus become much enlarged and elongate perpendicularly to the embryo sac. (Pls. 2, C, and 3, A.)

At anthesis the embryo sac has reached the mature egg stage. The egg is a large densely staining nucleated cell (Pl 1, C.) The synergids can be seen at the micropylar end of the embryo sac 12 hours after anthesis. (Pl 1, D) Further development of the embryo sac does not take place for more than 82 hours after anthesis. (Pl. 1, E, and pl. 2.) Development of the endosperm after this period is very rapid and is well ahead of the growth of the embryo (Pl. 3, A, B, C, D.) Within 94 to 130 hours after pollination definite walls have appeared in the endosperm. At 190 hours after anthesis the embryo has reached the stage where differentiation into the dermatogen, periblem, and plerome is just beginning (Pl. 3, F) The endosperm has continued to enlarge and lay down separating walls; at 190 hours after anthesis the endosperm has completely filled the embryo sac. (Pl 3, E.) At 224 hours after anthesis the embryo has grown very rapidly by division of the cells in all four tiers. The division lines between the four groups of cells are very clear. The dermatogen tissue of 1-cell thickness is very conspicuous and comes well down over the sides of the embryo. (Pl. 4, A) The inner cells of the integument appear to break down, and at this stage the endosperm does not completely occupy the embryo sac. At 237 hours after anthesis the embryo and endosperm are much larger, having grown very rapidly and the periblem initials and suspensor have appeared. (Pl. 4, B.)

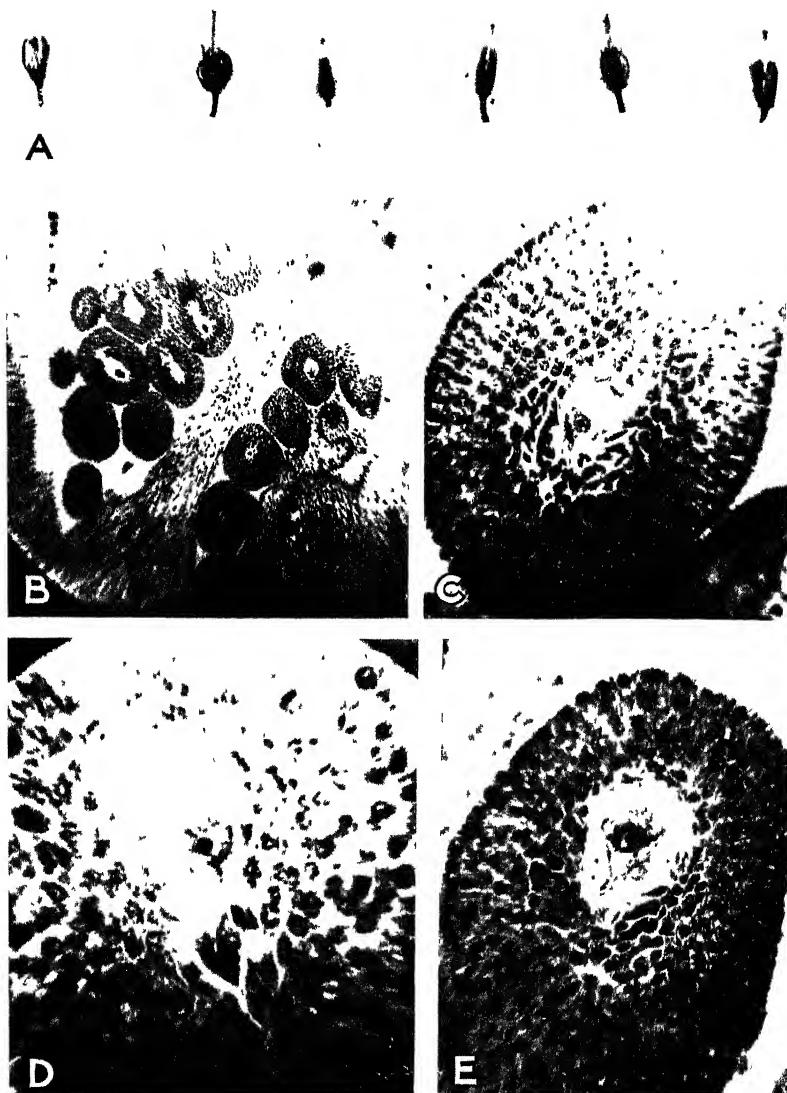
#### DEVELOPMENT OF OVULES AND EMBRYO SACS IN ABORTING PISTILS

The embryo sacs of aborting tomato pistils never developed beyond a weak mature egg stage. The embryo sac and ovules had not developed any farther at 190 hours after anthesis than they had at anthesis. (Pl. 4, C, D, E, F, G.) Other changes in the ovules and embryo sacs are characterized by a light-staining reaction of the cells, very small egg cell and nucleus, and a breaking down of the nucellus and cells of the integuments. The egg cell of the developing pistil is larger than that of the aborting pistil of the same age. (Compare pl. 2, B, with pl. 4, C, and pl. 3, A, with pl. 4, D.) Although the embryo sacs of the aborting pistils have not developed beyond the egg-cell stage at 190 hours after pollination, the embryo sacs of the developing pistils are completely filled with the many-celled endosperm and the embryo has developed at least to the 16-cell stage. (Compare pl. 3, F, with pl. 4, G.)

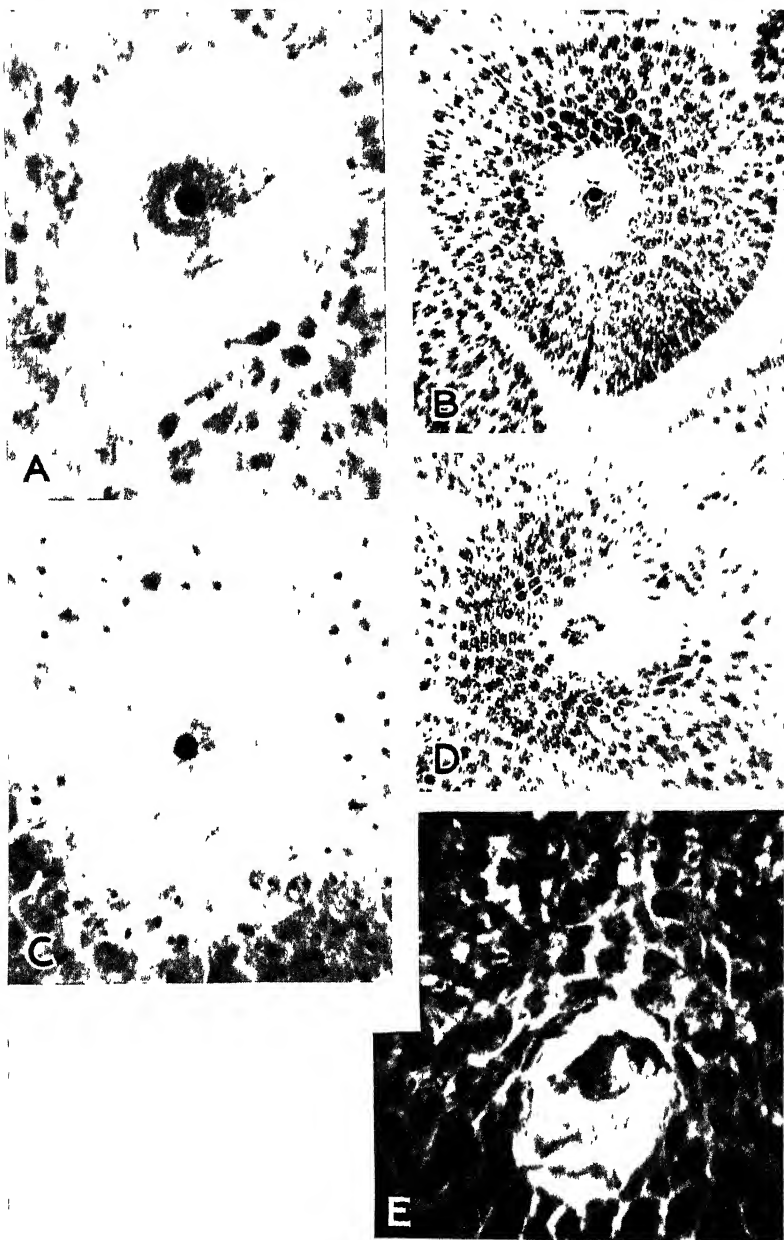
#### DISCUSSION

The abortion of the pistils studied was not due to lack of pollination, for all flowers were hand-pollinated. Growth of the ovules and embryo sac had ceased at the time of anthesis, when the flowers would normally be pollinated. Although the actual fertilization process was not observed in this study, initiation of growth of the embryo and endosperm was found to occur between 82 and 94 hours after pollination. This was determined by a comparison of the development of the embryo sacs at these two stages. The egg cell of the embryo sac in the aborting pistils was smaller at all times than that of developing pistils.

The results here shown do not preclude the contention that lack of pollination and fertilization or unfavorable conditions, such as hot dry winds, may cause pistil abortion and flower dropping. Observa-



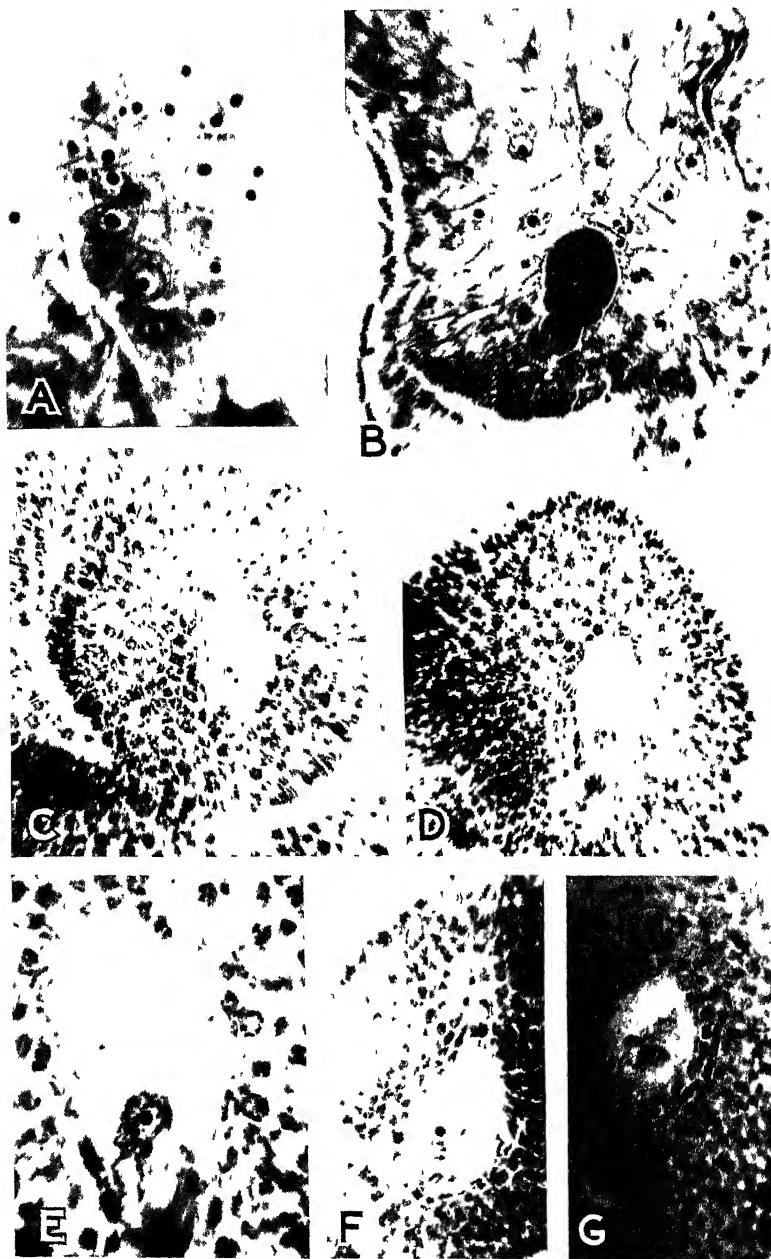
A, Normal tomato flower at the left, all others show abnormally elongated styles previous to anthesis,  $\times 34$ , B, portion of pistil, showing development of ovules at anthesis,  $\times 120$ , C, ovule and embryo sac 2 hours after pollination,  $\times 340$ , D, 12 hours after pollination,  $\times 650$ , E, 36 hours after pollination,  $\times 340$



Ovule and embryo sac of the tomato flower at various lengths of time after pollination. A, 36 hours,  $\times 650$ ; B, 54 hours,  $\times 340$ ; C, 60 hours,  $\times 650$ ; D, 70 hours,  $\times 340$ ; E, 82 hours,  $\times 650$



Ovule and embryo sac of the tomato flower at various lengths of time after pollination A, 94 hours and showing first indication of endosperm development,  $\times 340$ , B, 130 hours and showing embryo development,  $\times 340$ , C, 142 hours,  $\times 120$ , D, 151 hours,  $\times 340$ , E, 190 hours,  $\times 120$ , F, 190 hours,  $\times 340$ , G, 224 hours,  $\times 340$



A, Ovule and embryo sac of the tomato flower 224 hours after pollination,  $\times 650$ , B, ovule and embryo sac of the tomato flower 237 hours after pollination,  $\times 340$ , C, section of ovule of aborted pistil 50 hours after pollination,  $\times 340$ , D, aborted ovule 94 hours after pollination,  $\times 340$ , E, the same as D, 94 hours after pollination,  $\times 650$ , F, the same as D, 106 hours after pollination,  $\times 340$ , G, the same as D, 190 hours after pollination,  $\times 340$



Section of stigma and style showing pollen tube growth 24 hours after pollination,  
× 340



tions made at Oklahoma by the writer and also by Cross show that, even though pollinated, a great many flowers may drop during unfavorable weather. Plate 1, A, shows the reaction of style growth to low humidity, hot dry winds, and low soil moisture. Only the flower at the extreme left has a style of normal length. These flowers have not yet reached anthesis. Note the dark-colored stigmas which are drying up and collapsing, rendering pollen germination and pollen-tube growth unlikely.

Dorsey,<sup>9</sup> working with potato flowers, concluded that there are physiological influences operating independently of pollen or pistal development which cause the flowers to drop. He found evidences of disintegration in some embryo sacs, but on the whole they appeared to be undergoing the usual growth up to the time they were cut off when the flower dropped. Young<sup>10</sup> states that degeneration changes in the ovules and embryo sacs of potato flowers appear to result from unfavorable environmental conditions and may occur at any stage. In the late megaspore stage, blasting is accompanied by the shriveling of both the megaspore or embryo sac and the cells of the nucellus.

Pollen-tube growth in the tomato appears to be very slow. In Plate 5 it may be seen how short the growth of pollen tubes is 24 hours after pollination. This section shows only one twenty-fifth of the total length of the style. As pollen-tube growth is not initiated immediately after pollination, the actual growth shown in Plate 5 probably occurred in much less than 24 hours. Because of this slow growth of the pollen tube, the stigma, style, and pollen may be destroyed in periods of unfavorable weather before the pollen tubes reach the embryo sac. That this does occur is indicated by the high percentage of abortions observed when weather conditions are unfavorable. The plants from which these flowers were selected were grown in a greenhouse at temperatures ranging from 60° to 75° F. The soil moisture and the humidity of the atmosphere were favorable for good growth and fruit setting. The probable cause of the dropping of these flowers was the low nutrient supply in the soil or the inability of the plant to distribute properly and adequately the elaborated nutrients to the fast-developing embryo.

#### SUMMARY

The tomato plant has no one definite flowering peak. Flowering seems to be largely dependent upon soil moisture and temperature. The temperature existing approximately three days before anthesis appears to have the greatest influence upon flowering.

Blossom drop is greatly increased by hot dry winds and low humidity as well as by low soil moisture. On 10 plants under observation 47.4 per cent of the flowers aborted. There is a lag of approximately three days between the time that temperature exerts an effect on blossom drop and the time that the effect becomes visible.

During periods of hot dry winds and low soil moisture the styles elongate abnormally, even before anthesis. Few flowers that have elongated styles in hot dry weather develop normally and set fruit.

<sup>9</sup> DORSEY, M. J. A NOTE ON THE DROPPING OF FLOWERS IN THE POTATO. *Jour. Heredity* 10: 226-228, illus. 1919.

<sup>10</sup> YOUNG, W. J. THE FORMATION AND DEGENERATION OF GERM CELLS IN THE POTATO. *Amct. Jour. Bot.* 10: 325-335, illus. 1923.

At anthesis the embryo sac of the normal pistil had reached the mature egg cell stage. Under greenhouse conditions no further development of the embryo sac took place for more than 82 hours after anthesis. Initiation of growth of the embryo and endosperm occurred between 82 and 94 hours after pollination. At 190 hours after pollination the embryo had developed to the stage where differentiation into the dermatogen, periblem, andplerome was just beginning.

The embryo sacs of aborting pistils never developed beyond a very weak egg cell stage.

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## POLLEN ANTAGONISM IN COTTON<sup>1</sup>

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### INTRODUCTION

The writers (24)<sup>2</sup> have shown that when emasculated flowers of Pima Egyptian (*Gossypium barbadense* L.) or of upland cotton (*G. hirsutum* L.) are pollinated with a mixture of pollen from both types selective fertilization in favor of the like pollen results. The populations resulting from the double pollinations in the earlier experiments (24, Table I) yielded the data given in Table 1. From 67 to 82 per cent of the plants were of the type of the female parent, whereas, if there had been no selection among the pollen grains, only 50 per cent of the plants would have been of this type and the others heterozygous (Pima × upland or upland × Pima, F<sub>1</sub>). The departure from a 1:1 ratio was very significant in each of the populations, the magnitude of  $\chi^2$  indicates, in every case, chances more than 100 to 1 that the departure was not fortuitous.

TABLE 1—Evidence of selective fertilization when emasculated flowers of Pima Egyptian and of Lone Star and Acala upland cotton were pollinated with both kinds of pollen, as shown by the percentages of homozygous plants in the resulting populations

Female parent	Pollens applied *	Plants grown	Homozygous plants	$\chi^2$ of departures of numbers observed from numbers expected in the absence of selective fertilization
		Number	Per cent	
Pima .....	Pima and Lone Star .....	1,280	67.6±0.88	158.6
Lone Star .....	do .....	542	77.6±1.21	165.2
Pima .....	Pima and Acala .....	682	82.8±.97	293.5
Acala .....	do .....	618	67.7±1.27	77.5

\* In each of the 4 populations, one-half of the flowers were pollinated first with the like pollen and then with the unlike pollen, while on the other half the sequence was reversed. It was thought that combining the subpopulations from the 2 sequences of pollination would give an approximation to the results expected if the 2 pollens had been mixed together in equal quantities before being applied to the stigmas. Since the subpopulations representing the 2 sequences of pollination were of unequal size, instead of basing the percentage of homozygous plants, as given in this table, on the actual number of such individuals in the combined population, the average of the percentages of the 2 subpopulations was taken. In computing the probable error of this average percentage,  $n$  was taken as twice the number of plants in the smaller of the subpopulations. It is these modified numbers that are given under the heading "Plants grown."

Evidence was given in the paper cited (24) that the preponderance of homozygous individuals is not attributable to any lack of compatibility between these types of cotton or to differences in the viability of the two pollens or to selective survival at any stage after fertilization.

<sup>1</sup> Received for publication Sept. 21, 1931, issued April, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 224.

was accomplished. Indications also were obtained that such tubes of the unlike pollen as succeeded in penetrating the ovary had grown as rapidly as the tubes of the like pollen. Further investigation has yielded additional information on these points, which is given in the present paper.

A hypothesis formulated by the writers in attempting to explain the observed facts (24, p. 339) assumed that the like pollen stimulates a reaction in the tissues of the pistil, making the latter an unfavorable medium for the development of the unlike pollen. If this assumption is well founded, it follows that the more intimately the two pollens are in contact on the stigmas the greater the selective effect should be. The results of an experiment in which this test was applied are given in this paper. The question as to whether the like pollen must be present in a viable condition in order to exert an inhibiting effect upon the unlike pollen has been investigated also.

It has been shown by Jones (21) that in maize the degree of selective fertilization depends in large measure upon the degree of consanguinity of the two forms, usually being greatest between forms that are most distantly related. Experiments to determine whether this is the case in cotton, also, are described in this paper.

The experimental work was done at the United States field station, Sacaton, Ariz. In all experiments the flowers were emasculated in the evening before the day of anthesis and were pollinated the following morning. For information concerning the structure and ontogeny of the cotton flower, in relation to pollination and fertilization, the reader is referred to an earlier publication by one of the writers (22, *Pl. I-IV, VI, and VII*).

Pollen grains of the two types used in these experiments, Pima Egyptian and upland cottons, are shown in Figure 1.

#### POSSIBLE ALTERNATIVE EXPLANATIONS OF OBSERVED PHENOMENA

It is obvious, of course, that effects similar to those of selective fertilization would result from pollinations with mixed pollen if there were a difference in the compatibility of the two forms involved, or if the two pollens differed in viability, or if there were selective survival in favor of one or the other class of zygotes. Furthermore, selective fertilization might occur in the absence of any mutually antagonistic action of the two pollens if the rate of growth of the tubes differed consistently. The conclusion was reached that none of these explanations applied to the results previously published. This conclusion is supported by the results of the investigations reported herein.

#### CONDITIONS PRODUCING EFFECTS SIMILAR TO THOSE OF SELECTIVE FERTILIZATION

##### RELATIVE COMPATIBILITY OF THE POLLENS AS INDICATED BY DEGREES OF FERTILIZATION

Evidence was given in an earlier paper by one of the writers (22, *Table 23*) that pollen of upland cotton, when present alone on the stigmas of Pima Egyptian cotton flowers, effected fertilization in a degree equal or even superior to that effected by Pima pollen when present alone on the Pima stigmas. Additional data as to the mutual compatibility of these cottons have been obtained in later experiments, the results of which are summarized in *Table 2*.

TABLE 2—*Mutual compatibility of the two types of cotton as shown by degrees of fertilization effected by pollen of Pima Egyptian and of upland cotton (Lone Star and Acala varieties) when applied separately to stigmas of one or the other type, measured by the percentage of bolls retained and the mean number of seeds per boll*<sup>a</sup>

Year	Experiment No	Pollination	Flowers pollinated	Bolls retained	Seeds per boll
			Number	Per cent	Mean number
1922	1	Pima × Pima.....	99	99 0±0 67	15 6±0 24
		Pima × Lone Star.....	100	96 0±1 32	17 0± 20
		Difference.....		3 0±1 48	1 4± 31
		Lone Star × Lone Star.....	99	22 2±2 82	30 1±1 14
		Lone Star × Pima.....	100	12 0±2 19	24 3±1 30
		Difference.....		10 2±3 57	5 8±1 73
1922	3	Pima × Pima.....	50	92 0±2 59	15 3± 38
		Pima × Lone Star.....	50	82 0±3 66	17 6± 36
		Difference.....		10 0±4 49	2 3± 52
		Lone Star × Lone Star.....	49	49 0±4 81	33 3± 88
		Lone Star × Pima.....	50	42 0±4 72	31 5± 69
		Difference.....		7 0±6 75	1 8±1 12
1925	7	Pima × Pima.....	93	92 5±1 84	15 6± 22
		Pima × Acala.....	97	94 8±1 52	16 7± 25
		Difference.....		2 3±2 39	1 1± 33
1927	2	Pima × Pima.....	100	65 0±3 22	16 2± 23
		Pima × Acala.....	100	65 0±3 22	17 5± 24
		Difference.....		0 ±4 55	1 3± 33
1928	1	Pima × Pima.....	101	97 0±1 15	18 0± 21
		Pima × Acala.....	100	93 0±1 72	18 2± 23
		Difference.....		4 0±2 07	2± 31
		Acala × Acala.....	50	98 0±1 33	33 0± 25
		Acala × Pima.....	50	96 0±1 87	31 1± 51
		Difference.....		2 0±2 30	1 9± 57

<sup>a</sup> The much heavier rate of boll shedding in upland as compared with Egyptian cottons is reflected in the much lower percentages of bolls retained from pollinations on the upland plants in the experiments of 1927. The effect of removal of most of the early flowers is seen in the high percentage of bolls retained on the Acala plants in the experiment of 1928. The mean number of seeds per boll is, of course, much greater in the large 4-lock or 5-lock upland bolls than in the small, mostly 3-lock, Pima bolls.

The data in Table 2 show no significant differences for either cotton in the percentages of bolls retained from pollination with like and with unlike pollen. As indicated by this criterion, the two pollens are equally efficient when applied separately to the stigmas of either type. In all the experiments except that of 1928, in which there was practically no difference, the mean number of seeds in the Pima bolls was significantly greater when the flowers were pollinated with the unlike (upland) pollen than when they were pollinated with the like (Pima) pollen. In the three experiments in which the results of pollinations on upland plants were compared, the mean number of seeds in the upland bolls was greater when the flowers had been pollinated with the like (upland) pollen than when they had been pollinated with the unlike (Pima) pollen, and the difference was significant in two of these experiments.

So far as may be concluded from the data at hand, it appears that on the Pima stigmas the fertilizing ability of the unlike (upland) pollen usually is somewhat greater than that of the like (Pima) pollen and

that on the upland stigmas there is usually a difference in favor of the like (upland) pollen. As a result of these differences, the action of selective fertilization in favor of the like pollen would be partly counteracted in the Pima flowers and somewhat exaggerated in the upland flowers. Somewhat greater viability of the upland pollen seems to be indicated by these comparisons, although, as will be shown in the following section, tests in sugar solution have shown no consistent difference in the viability of the two kinds of pollen.

#### RELATIVE VIABILITY OF THE POLLENS AS INDICATED BY BEHAVIOR IN SUGAR SOLUTIONS

No very satisfactory artificial medium for the germination of cotton pollen has been devised (2). Observation of the germination of the pollen when placed on the stigmas of cotton flowers is difficult because the pollen tubes can not be distinguished readily from the hyaline projections of the stigmatic surface. It was discovered by A. E. Longley<sup>3</sup> that pollen of Pima and of upland cotton germinates readily when placed on corn silks, although care must be taken not to have the surface too moist, as otherwise the grains burst. Doctor Longley noted that both on corn silks and in an artificial medium of 2 per cent agar + 15 per cent cane sugar the tendency to bursting is greater in Acala upland pollen than in Pima pollen. He also observed that the tubes from the smaller upland pollen grains are only about half the diameter of the Pima pollen tubes and grow much more rapidly than the latter in this medium.

Observation during several seasons at Sacaton, Ariz., has shown that upland cottons produce much nonviable pollen during the early part of the summer and during periods of high humidity, while throughout the season the pollen of Pima cotton usually contains but a small proportion of nonviable grains. This difference would have seriously impaired the results of experiments in selective fertilization had not the precaution been taken of deferring such experiments until observation indicated that the viability of the two pollens had become approximately equal.

Tests of pollen viability have been made in a 5 per cent solution of cane sugar, in which medium, and also in distilled water, the grains explode suddenly instead of germinating slowly as they do when placed on cotton stigmas. The term "pseudogermination" was applied by Andronescu (1) to this sudden extrusion of the contents of the grain (22, p. 22-25). It seems justifiable, however, to regard the proportion of the total number of grains in the field of the microscope that extrude their contents in these media as an indication of the relative viability, because it is the abnormally small and the abnormally large grains that fail to explode.

In the earlier experiments the viability of the two pollens, Pima and upland, was tested by the method just described (24, p. 332), and no significant difference was detected. Similar tests were made in connection with some of the experiments described in the present paper and will be considered in relation thereto. No indications were obtained of differences in viability of such magnitude as to impair the evidence in favor of selective fertilization.

<sup>3</sup> Unpublished work

## DIFFERENTIAL SURVIVAL OF HOMOZYGOTES AND HETEROZYGOTES

The criterion of selective fertilization used in all the experiments has been the percentage of homozygous (like  $\times$  like) individuals in the resulting population. This criterion would be open to serious objection if there were differential survival of either class of plants at germination or in some later stage of growth. If the homozygous individuals survived in relatively greater number, the effect would be the same as that of selective fertilization in favor of the like pollen. On the other hand, better survival of the heterozygous plants would counteract the effect of selective fertilization in favor of the like pollen or, if the difference in rate of survival were great enough, it might create the appearance of selective fertilization in favor of the unlike pollen.

Determinations of weight and of percentage of germination were made on the seeds resulting from separate application of the two pollens in experiment 1 of 1922 (Table 2). The results, summarized in Table 3, indicate that the heterozygous seeds, obtained by fertilization with unlike pollen, were somewhat heavier and germinated better. The difference in percentage of germination was of considerable magnitude between the seeds from upland flowers pollinated with like and with unlike pollen, respectively. It may be assumed, therefore, that whatever differential survival occurs at germination favors the heterozygotes and prevents the full expression of selective fertilization in favor of the like pollen.

TABLE 3—*Relative germination of seeds obtained by pollinating flowers of Pima Egyptian and of Lone Star upland cotton with pollen of each type separately*

Pollination	Mean weight of 50 seeds *	Mean germination
Pima $\times$ Pima.....	Grams 6 44 $\pm$ 0 03	Per cent 85 9 $\pm$ 1 08
Pima $\times$ upland .....	6 76 $\pm$ 03	91 0 $\pm$ 86
Difference.....	32 $\pm$ 04	5 1 $\pm$ 1 38
Upland $\times$ upland.....	6 29 $\pm$ 05	57 2 $\pm$ 2 11
Upland $\times$ Pima.....	6 76 $\pm$ 20	74 4 $\pm$ 1 86
Difference.....	47 $\pm$ 21	17 2 $\pm$ 2 81

\* The weights were determined on lots of 50 seeds each, distributed among the several pollinations as follows: Pima  $\times$  Pima, 30, Pima  $\times$  upland, 32, upland  $\times$  upland, 13, upland  $\times$  Pima, 5. The means were computed from the weights of the several lots representing each pollination.

In connection with the experiments on selective fertilization performed in 1922 and described in an earlier publication, consideration was given to the question whether there had been differential survival at any stage from germination to the time when the plants were large enough to distinguish with certainty between the homozygotes and the heterozygotes. The conclusion was reached that selective survival at or after germination, as indicated by comparing the percentages of hybrids where the conditions were more favorable and less favorable to the growth of the plants, had not been an important factor in bringing about the observed results. Indirect evidence was presented that the results are not attributable to selection between the homozygotes and heterozygotes at any stage between fertilization and germination (24, p 333-335).

At the time these experiments were performed the soil of the Sacaton station was only very locally and nowhere seriously infested

with nematodes. Subsequently, however, pronounced infestation took place. It has been found at this station that cotton of the Egyptian type is much more susceptible than upland cotton to injury by these organisms, which frequently cause heavy mortality among the plants of Pima and other Egyptian varieties, especially in the seedling stage. It has been observed, also, that the Pima  $\times$  upland  $F_1$  plants are less susceptible to such injury than the Pima plants, although more so than the upland plants. There is good reason to believe that selective survival of the heterozygous plants, due to this factor, has invalidated some of the results obtained in recent years, as will be discussed in connection with the experiments involved.

#### DIFFERENTIAL GROWTH RATE OF POLLEN TUBES AS A CAUSE OF SELECTIVE FERTILIZATION

The occurrence in cotton of selective fertilization in favor of like pollen is believed to have been proved by the evidence that differences in compatibility and viability of the pollens and rate of survival do not account for the observed excess of homozygous plants in the populations from mixed pollinations. The question next to be considered is whether the selection is due to differential growth rate of the pollen tubes.

Evidence has been obtained by Jones and other investigators (21 p. 10-34) that in maize and other plants selective fertilization is due to differences in the rate of development of the two pollens, conditioned, presumably, by genetic differences in the determiners for pollen-tube growth. Direct observation of growth of the pollen tubes in the pistils of the cotton plant has not been attempted, but two indirect methods of calculating the relative rates of growth have been employed. The first method involved the pollination of different individual flowers, some with like and some with unlike pollen. The pistils were excised at the summit of the ovary at stated intervals after the pollen was deposited, and the degrees of fertilization attained in each period with each kind of pollen were compared. This will be referred to hereafter as the excision method.<sup>4</sup>

The second method, used by Correns in his studies of selective fertilization in *Melandrium* (*Lychnis*), assumes that in plants whose ovaries contain many ovules vertically superposed, if two pollens are applied that differ in their rate of tube growth, the faster growing pollen will fertilize the upper ovules first and if the quantity of pollen is limited the lower ovules will be left to fertilization by the slower-growing kind. If the resulting seeds from the upper and the lower half of the capsule are planted separately, a higher percentage of hybrids from the lower seeds than from the upper seeds would therefore indicate that the tubes of the unlike pollen had grown more slowly than the tubes of the like pollen, and vice versa.<sup>5</sup>

<sup>4</sup> This method was used in studies of *Oenothera* by Heribert-Nilsson (18, 19) and of *Melandrium* by Correns (3). The term "certation" (Zertation) was used by Heribert-Nilsson to designate differential growth rate of the pollen tubes. Harland (14) suggests the term "agonisis," meaning a struggle, for this phenomenon.

<sup>5</sup> Correns (3, p. 338-341) pollinated flowers of a recessive white-flowered race of *Melandrium rubrum* Garcke (*Lychnis dioica* L.) first with pollen of a dominant red-flowered race and several hours later with pollen of the white-flowered race. The seeds from the upper and the lower part of the resulting capsules were planted separately and there was a much higher percentage of red-flowered individuals in the population from the upper seeds than in the population from the lower seeds. He concluded that the pollen tubes that enter the ovary first tend to fertilize the upper ovules. A similar method was used by Huorth (20) in studies of differential pollen-tube growth in *Oenothera*.

## GROWTH RATE TESTED BY EXCISION METHOD

## POLLENS APPLIED SEPARATELY

In an experiment (No 3) performed in 1922, flowers on plants of Pima and of Lone Star upland cotton were emasculated in the evening and pollinated the following morning, some with the like and some with the unlike pollen, giving four different pollinations Pima  $\times$  Pima, Pima  $\times$  upland, upland  $\times$  upland, and upland  $\times$  Pima. The flowers representing each pollination were then divided into lots of approximately 50 each, and the styles and stigmas of the several lots were removed, by excision at the summit of the ovary, at successive intervals. The excisions were made at 5, 8, and 11 p. m. of the day of pollination and at 5 a. m. of the day following, corresponding, respectively, to the following number of hours after the pollen was applied 8, 11, 14, and 20. In an approximately equal number of flowers representing each pollination the styles and stigmas were not excised, and these flowers served as controls. The number of flowers of a given pollination, excised at a given hour or left without excision, was in no case fewer than 44. The bolls developing from these several treatments were collected when they matured, and record was made of the number of bolls from each lot of flowers and of the number of seeds in each boll. From these data the statistical constants in Table 4 were computed.

The results from excision of the styles and stigmas at successive intervals, as given in Table 4, show that while the ovaries of a few of the Pima flowers had been penetrated within 8 and 11 hours after pollination by enough pollen tubes to insure retention of the boll, no bolls were retained by the upland flowers excised at these intervals. This indicates a slower rate of growth of the pollen tubes in the upland pistils, especially as the average length of the latter above the summit of the ovary is only about 60 per cent of the average length of the corresponding portion of the Pima pistils. Even when excision was deferred until 14 hours after pollination, the percentage of bolls retained, relative to that of the respective controls, was much greater from the Pima than from the upland flowers; but the number of seeds per boll, relative to that of the respective controls, indicated better fertilization of the upland than of the Pima flowers during this period.

A comparison of the two pollens as to relative growth of their tubes in the pistils of each kind of cotton shows that the rate of penetration of the ovary, as measured by the fertilization attained, was nearly the same for both pollens, in both the Pima and the upland flowers. Neither the percentage of bolls retained nor the mean number of seeds per boll differed significantly between the two pollinations on flowers of either type, with three exceptions. Bolls matured from 2 of the 49 Pima flowers pollinated with Pima pollen and excised after 8 hours, whereas no bolls matured from the upland flowers pollinated with Pima pollen and excised at the same time. Also, in bolls from Pima flowers excised after 11 hours and from those not excised, pollination with upland pollen gave a significantly greater mean number of seeds than pollination with Pima pollen.<sup>6</sup> So far as may be judged by the

<sup>6</sup> When comparison is made of the results from excision after 14 hours with the results from the unexcised (control) flowers the relative mean number of seeds after 14 hours is seen to be considerably greater from the pollination Pima  $\times$  Pima than from the pollination Pima  $\times$  upland. As percentages of the numbers from the respective controls, the values are  $56.2 \pm 4.1$  and  $39.7 \pm 4.3$ . The probable errors were computed as the probable error of the quotient  $\frac{\text{Mean number of seeds from 14-hour excision}}{\text{Mean number of seeds from control}} \times 100$ . The difference, however, is hardly significant, being only 2.8 times its probable error.

results obtained by this method, there is, however, no important and consistent difference in the rate of growth of the tubes of the two kinds of pollen in the pistils of either type of cotton.

TABLE 4.—*Relative completeness of fertilization, as indicated by the percentage of bolls retained and the mean number of seeds per boll, in flowers of Pima Egyptian and Lone Star upland cotton, pollinated with like and with unlike pollen, from which the styles and stigmas had been removed by excision at successive intervals or had not been removed*<sup>a</sup>

[Experiment No. 3, 1922]

Interval between pollination and excision	Pollination	Bolls retained	Seeds per boll
		<i>Per cent</i>	<i>Mean number</i>
8 hours	Pima × Pima	4.1 ± 1.89	14.0 ± 2.86
	Pima × upland	0	0
	Difference	4.1 ± 1.89	14.0 ± 2.86
	Upland × upland	0	0
	Upland × Pima	0	0
	Difference		
11 hours	Pima × Pima	14.0 ± 3.31	4.9 ± 1.58
	Pima × upland	10.0 ± 2.86	13.2 ± 1.84
	Difference	4.0 ± 4.40	8.3 ± 2.42
	Upland × upland	0	0
	Upland × Pima	0	0
	Difference		
14 hours	Pima × Pima	60.4 ± 4.77	8.6 ± .59
	Pima × upland	54.0 ± 4.75	7.0 ± .74
	Difference	6.4 ± 6.74	1.6 ± .95
	Upland × upland	8.3 ± 2.68	27.2 ± 3.35
	Upland × Pima	14.0 ± 3.31	25.3 ± 1.87
	Difference	5.7 ± 4.26	1.9 ± 3.84
20 hours	Pima × Pima	76.0 ± 4.07	15.0 ± .57
	Pima × upland	78.0 ± 3.95	16.3 ± .45
	Difference	2.0 ± 6.68	1.3 ± .73
	Upland × upland	15.9 ± 3.73	32.1 ± 1.59
	Upland × Pima	18.8 ± 3.81	32.9 ± 1.30
	Difference	2.9 ± 5.31	8 ± 2.06
Control (not excised)	Pima × Pima	92.0 ± 2.59	15.3 ± .38
	Pima × upland	82.0 ± 3.66	17.6 ± .36
	Difference	10.0 ± 4.48	2.3 ± .52
	Upland × upland	49.0 ± 4.81	33.3 ± .88
	Upland × Pima	42.0 ± 4.72	31.5 ± .69
	Difference	7.0 ± 6.74	1.8 ± 1.12

<sup>a</sup> The heavier rate of boll shedding, characteristic of upland as compared with Pima cotton, is indicated by the much lower percentages of bolls retained from the upland flowers in comparison with those retained from the Pima flowers. The mean numbers of seeds per boll are of course greater in the large 4-lock and 5-lock upland bolls than in the small, mostly 3-lock Pima bolls.

Great variation in the rate of growth of individual pollen tubes is indicated by the data in Table 4. In a few of the Pima flowers the number of tubes that penetrated the ovary within 8 hours after pollination was sufficient to insure retention of the boll, but 20 hours were required to effect a degree of fertilization approximating that of the control flowers. Taking 33 mm. as the average length of the Pima

pistils from the summit of the stigmas to the summit of the ovary, the indicated variation in the mean hourly growth rate of the pollen tubes was from 1.65 mm (33/20) to 4.12 mm (33/8). In the upland flowers a sufficient number of pollen tubes to insure retention of the boll had not penetrated the ovary within 11 hours after pollination. Assuming that at least 12 hours is required for penetration, and taking 20 mm as the average length of the Lone Star upland pistils, the most rapid growth rate of the pollen tubes in the upland cotton pistils apparently did not exceed 1.67 mm (20/12) per hour, which is practically the same as the minimum computed for the rate of growth in Pima pistils.<sup>7</sup>

TABLE 5—*Relative completeness of fertilization, as indicated by the percentage of bolls retained and the mean number of seeds per boll, in flowers of Pima Egyptian cotton, pollinated with like and with unlike pollen, from which the styles and stigmas had been removed by excision at successive intervals or had not been removed*

[Experiment No. 7, 1925]

Pollination	Bolls retained and mean number of seeds after indicated interval between pollination and excision					
	12 hours		21 hours		Control (not excised)	
	Bolls retained	Seeds	Bolls retained	Seeds	Bolls retained	Seeds
	Per cent	Mean number	Per cent	Mean number	Per cent	Mean number
Pima × Pima.....	23 5±2 88	5 0±0 67	81 2±2 69	13 3±0 30	92 5±1 84	15 0±0 22
Pima × upland.....	27 3±3 02	5 8± 40	82 6±2 58	15 4± 25	94 8±1 52	16 7± 25
Difference.....	3 8±4 17	8± 78	1 4±3 73	2 1± 41	2 3±2 39	1 1± 33

In a similar experiment (No. 7), performed in 1925, flowers on Pima plants were emasculated, and half of them were pollinated with Pima pollen and half with pollen of Acala upland cotton. In approximately equal numbers of flowers of each pollination the styles and stigmas were excised at intervals of 8, 10, 12, and 21 hours after the pollen was applied; a fifth lot was left without excision, as a control. Each lot comprised from 93 to 100 flowers. There was no fertilization of the flowers excised 8 hours after pollination, and the number of bolls retained from flowers excised after 10 hours was only 2 in the population Pima × Pima and 5 in the population Pima × upland. The results from the later excisions and from the controls are given in Table 5.

<sup>7</sup> The length of the pistil from the summit of the stigmas to the apex of the ovary was measured at Sacaton in 1922 on 50 flowers each of Pima Egyptian and Lone Star upland cotton and in 1923 on 50 flowers each of Pima and of the Lone Star and Acala varieties of upland cotton. The means obtained were as follows (millimeters):

	Pima	Lone Star	Acala
1922.....	32 2±0 16	17 2±0 11	—
1923.....	34 1± 11	22 6± 17	23 4±0 15
Average.....	33 2	19 9	—

Determinations of the distance from the apex of the ovary to the uppermost ovule gave the following means: 2.9 ± 0.03 mm for Pima (15 flowers) and 2.3 ± 0.08 mm for Lone Star (10 flowers). Since in the experiments described in this section the excisions were made at the apex of the ovary, only the distance from the summit of the stigmas to that point need be considered.

Under natural conditions the time available for penetration of the ovary is limited by the number of hours from the opening of the corolla and deposition of pollen to the abscission of the pistil at the summit of the ovary when the flower withers. The mean length of this period, as determined at Sacaton in 1922 on 50 Pima flowers, was 29 hours. It has been observed that it is somewhat longer in humid, cloudy weather and shorter in dry, sunny weather.

The percentages of bolls retained from the two pollinations did not differ significantly in any of the subpopulations, but from flowers of which the styles were excised 21 hours after pollination and from the unexcised (control) flowers the mean number of seeds was significantly higher in bolls from flowers pollinated with unlike pollen (Pima  $\times$  upland) than in bolls from flowers pollinated with like pollen (Pima  $\times$  Pima). The mean differences were 5.1 and 3.3 times their respective probable errors. These differences suggest a difference in the viability of the two pollens. Tests in sugar solution of the pollens used in this experiment indicated that such was the case, as the pollen from several Pima flowers contained from 20 to 50 per cent of defective grains, while the number of such grains in the samples of upland pollen was negligible. There was, however, no appreciable difference in the rapidity and completeness of explosion of the normal grains of the two pollens. The pollinations were made with such a large excess of pollen grains that it is improbable that the content of defective grains in the Pima pollen was an important factor in the result.

TABLE 6—Differences between Pima and upland cotton in rate of pollen-tube growth, as indicated by the increase in mean number of seeds per boll from the control flowers excised later or not at all over the mean number of seeds per boll from flowers excised earlier

Year	Experiment No.	Pollination	Interval between pollination and excision		Increase in mean number of seeds in the controls
			Shorter	Longer	
1920	a5	Pima $\times$ Pima	16½	24½	2.9±0.78
		Pima $\times$ upland	16½	24½	8±1.06
		Difference			2.1±1.32
1922	b3	Pima $\times$ Pima	14	(c)	6.7±.70
		Pima $\times$ upland	14	(c)	10.6±.82
		Difference			3.9±1.08
1922	b3	Upland $\times$ upland	14	(c)	6.1±3.46
		Upland $\times$ Pima	14	(c)	6.2±1.99
		Difference			1±1.00
1925	a7	Pima $\times$ Pima	12	(c)	10.6±.71
		Pima $\times$ upland	12	(c)	10.9±.47
		Difference			3±.85

<sup>a</sup> The data of this experiment are given in another paper (22, Table 24). No flowers were left unexcised, so flowers excised 24½ hours after pollination are taken as the controls.

<sup>b</sup> Data given in Table 4 of this paper.

<sup>c</sup> Not excised.

<sup>d</sup> Data given in Table 5 of this paper.

Table 6, summarizing the results of several experiments, shows a comparison of the degrees of fertilization attained by applying the two pollens separately and allowing a shorter and a longer interval for the pollen tubes to penetrate the ovary. The data indicate in only one comparison a significant difference between the two types of cotton in the rate of growth of the pollen tubes. In the experiment of 1922 the increase in the mean number of seeds from the unexcised

control flowers over the mean number from the flowers excised 14 hours after pollination was decidedly greater for the Pima flowers fertilized with upland pollen than for the Pima flowers fertilized with Pima pollen. The difference of 3.9 seeds between the mean increases may be considered significant, being 3.6 times its probable error. In this case the fact that fertilization was more nearly complete after 14 hours in the flowers that received pollen of their own variety than in the flowers that received pollen of another species (upland) indicated more rapid growth of the tubes of the like pollen.

It may not be concluded, however, from the evidence in Table 6 that more rapid growth of the tubes of the like pollen in the Pima pistils is the rule. In the experiment of 1920 the comparison indicated more rapid growth of the unlike pollen, although the difference was not significant and in the experiment of 1925 there was practically no difference between the two pollens. The only comparison of the rate of growth of the tubes of different pollens in pistils of upland cotton (upland  $\times$  upland and upland  $\times$  Pima in the experiment of 1922) gave no evidence of a difference in rate of growth of the like and the unlike pollen tubes.

#### POLLENS APPLIED MIXED

It has been seen that excision experiments in which the two kinds of pollen were applied separately gave no evidence of a consistent difference in the growth rate of the tubes. It does not follow, however, that the growth rates are the same when a mixture of both pollens is presented to the stigmas of the same individual flower. To test this point, an experiment was made in 1925 in which Pima flowers were emasculated in the evening and pollinated the following morning at 9 a. m. with an intimate mixture of approximately equal quantities of Pima pollen and Acala upland pollen. The pistils of equal numbers of these flowers were then excised at the summit of the ovary at intervals of 9, 11, 13, 20, and 24 hours, respectively, after pollination, a sixth lot was left unexcised, as a control.

Tests in sugar solution of pollen from five flowers of each variety, made at the beginning of the experiment, indicated a slight difference in viability in favor of the upland pollen. The estimated percentage of small and doubtless defective grains varied from 5 to 10 per cent in the several Pima flowers and from 2 to 5 per cent in the several upland flowers. In each lot of pollen there were also a few abnormally large grains, which failed to explode or exploded only after very long immersion. There appeared to be no difference between the Pima and upland pollens in the rapidity and completeness of explosion of the normal-sized grains.

This experiment was planned on the assumption that if the tubes of the like (Pima) pollen reached the ovary sooner than the tubes of the unlike (upland) pollen, the percentage of homozygous individuals in populations grown from the resulting seeds would be greater in populations from the earlier-excised flowers than in populations from flowers excised later or not excised. Only 2 or 3 bolls each matured from the lots of flowers that were excised 9 and 11 hours after pollination. The seeds produced by the remaining lots of flowers were planted in 1926, and the percentages of homozygous individuals in these populations are given in Table 7.

TABLE 7—Percentages of homozygous (*Pima*) plants in populations from seeds produced by *Pima* flowers which were pollinated with a mixture of *Pima* and upland pollens and from which the styles and stigmas had been removed by excision 13, 20, and 24 hours after pollination, or had not been removed

[Experiment No 6, 1925]

Interval between pollination and excision (hours)	Plants grown	Homozygous plants	$\chi^2$ of departures of numbers observed from numbers expected in the absence of selective fertilization
	Number	Per cent	
13.....	83	81 $9 \pm 2$ 85	33 8
20.....	692	88 $2 \pm$ 83	403 8
24.....	654	90 $7 \pm$ 76	536 6
Control (not excised).....	627	87 $2 \pm$ 90	346 8

Contrary to the assumption, the population from the flowers excised earliest contained the lowest percentage of homozygous plants, indicating more rapid growth of the unlike (upland) pollen tubes. The percentage for the 13-hour excision does not, however, differ significantly from any of the others except, possibly, from the percentage for the population from flowers excised after 24 hours, and in this case the difference is only three times its probable error. It must be concluded that this experiment gave no satisfactory evidence of a difference in the growth rate of the two pollens when both are present on the same stigmas.

A very pronounced degree of selective fertilization in favor of the like pollen is shown by the data in Table 7, notwithstanding the somewhat greater viability of the upland pollen indicated by the tests in sugar solution. The departures, in the several populations, from the 50 per cent of homozygous plants expected if there had been no selective fertilization were, respectively, 7, 25, 27, and 23 times the probable error of the departure. The magnitude of  $\chi^2$  of the departures of the observed numbers of homozygous and heterozygous plants from the numbers expected had there been no selective fertilization indicates, in every case, chances many more than 100 to 1 that the departure is significant.<sup>8</sup> Since the slight difference in viability of the two pollens indicated by the results of tests in a sugar solution was in favor of the upland pollen and the pollinations were made on *Pima* flowers, the tendency of this difference would have been to obscure rather than to magnify the effect of selective fertilization.

#### GROWTH RATE TESTED BY COMPARING POPULATIONS FROM UPPER AND LOWER SEEDS

Another method for determining whether there is differential growth rate of the tubes from like and from unlike pollen is to apply to the stigmas a mixture of the two kinds of pollen and to determine the proportion of homozygous plants in populations grown from the

<sup>8</sup> It is noteworthy that the percentage of homozygous plants in the population from the unexcised (control) flowers in this experiment is almost the same as in the comparable population C of experiment 5 grown the same year from flowers pollinated in the same manner as in the experiment here described, and not excised (Table 9).

upper and from the lower seeds in the resulting bolls. This method is based on the assumption that the apical ovules are likely to be fertilized by the pollen tubes that enter the ovary first.<sup>9</sup> In that case the populations would be expected to differ in the percentage of homozygous plants, the percentage being greater in the population from upper seeds if the like pollen grew more rapidly and in the population from lower seeds if the unlike pollen grew more rapidly. In the experiments described in an earlier paper (24, p 337, 338) application of this method showed no significant differences between the populations from upper and from lower seeds, but this may have been due to the fact that the number of pollen grains placed on the stigmas was many times greater than the number of ovules.

With a large excess of pollen, the tendency would be to fertilization of all or nearly all the ovules by the more rapidly growing kind of pollen, and there would be less likelihood of a difference in the proportion of homozygotes and heterozygotes between the populations from upper and from lower seeds. On the other hand, if the number of pollen grains in the mixture does not greatly exceed the number of ovules, fertilization of the upper ovules by the more rapidly growing pollen and of the lower ovules by the slower-growing pollen may be expected.<sup>10</sup> Experiments with pollen mixtures, therefore, were undertaken in which the total number of pollen grains applied to the stigmas was not greatly in excess of the number of ovules.

In an experiment (No 6) performed in 1926, 15 grains of Pima pollen and 15 grains of Acala upland pollen were mixed together and the mixture was applied to the stigmas of 75 emasculated Pima flowers. Thus, 30 grains of pollen were applied to each flower, although the average number of ovules in the ovary of the Pima flower is approximately 21 (22, p 51). The excess of 9 grains was intended to compensate for possible lack of viability in a few of the pollen grains. Bolls were retained from 62 flowers (82.7 per cent of the number treated) and the mean number of seeds in these bolls was 7.7, whereas in previous experiments in which much larger quantities of pollen were applied to emasculated Pima flowers, the mean number of seeds per boll was from 16.6 to 17.0. It may be concluded that the 30 grains of pollen used in the present experiment were too few to give the maximum degree of fertilization attainable in emasculated and artificially pollinated flowers.

The bolls that matured were divided into upper and lower halves and the seeds from each half were planted separately in 1927 to determine the percentages of homozygous plants. The resulting data, given in the upper section of Table 8, show no evidence of a difference in rate of growth of the two kinds of pollen, the difference between the percentages of homozygous individuals in the two subpopulations having only slightly exceeded the probable error of the difference. Moreover, there was no evidence of selective fertilization,

<sup>9</sup> That this may be the case in cotton is indicated by data obtained by Rea (29), who found that in upland cotton bolls the proportion of motes or unfertilized ovules increases from the top to the bottom of the boll.

<sup>10</sup> Correns (8) found that in the dioecious *Melandrium rubrum* the percentage of pistillate plants in the resulting progeny was greater when the flowers received a large excess of pollen than when a smaller number of pollen grains was applied. He concluded from this fact that the tubes of the pistillate-determining grains grow faster than the tubes of the staminate-determining grains. References to earlier papers of this author are given in the publication cited. The possibility of distortion of Mendelian ratios as a result of differential growth rate of the tubes of genetically different pollen grains seems first to have been suggested by Renner (30), who later (31) obtained different ratios in *Oenothera* depending upon whether few or many pollen grains were used. The assumption was that when pollen is used sparingly the slower-growing tubes have a chance to fertilize more of the ovules.

the percentage of homozygous plants in the combined population not having departed significantly from 50 per cent. The probable reason for the apparent absence of selective fertilization will be considered presently.

TABLE 8—Numbers of plants and percentages of homozygous plants in populations grown from seeds from the upper half and from the lower half of bolls produced by *Pima* flowers pollinated with mixtures of equal numbers of grains of *Pima* and upland pollen

EXPERIMENT OF 1926

Pollen grains applied		Plants from—			Homozygous plants from—			χ <sup>2</sup> of departures of numbers observed from numbers expected in the absence of selective fertilization (from all seeds)
Each kind (number)	Total	Upper seeds	Lower seeds	All seeds	Upper seeds	Lower seeds	All seeds	
15 -----	Number 30	Number 137	Number 155	Number 292	Per cent 45.3±2.87	Per cent 49.7±2.71	Per cent 47.6±1.97	0.67

EXPERIMENT OF 1927

15 -----	30	272	295	567	57.0±2.02	57.0±1.94	57.0±1.40	11.12
20 -----	40	258	171	429	49.7±2.10	52.7±2.57	50.8±1.63	11
25 -----	50	78	125	203	50.0±3.82	48.8±3.01	49.3±2.37	04
30 -----	60	156	182	338	48.7±2.69	47.8±2.50	48.2±1.83	44

In a similar experiment (No. 1) performed in 1927, four lots of emasculated *Pima* flowers were pollinated with mixtures of 15, 20, 25, and 30 grains each of two kinds of pollen, *Pima* and *Acala* upland, hence with totals of 30, 40, 50, and 60 grains, respectively. There were no significant differences among the several lots of bolls obtained in respect to the mean number of seeds, hence no evidence that 60 grains of pollen effected more nearly complete fertilization than was attained with 30 grains. The mean number of seeds having ranged from 11.9 to 13.3 in the several lots, the degree of fertilization was in all cases much higher than was attained with 30 grains of pollen in the experiment of 1926 but fell considerably short of the degree attained in earlier selective-fertilization experiments in which each flower doubtless received many more than 60 pollen grains.<sup>11</sup>

The seeds obtained by pollination with the several pollen mixtures were planted in 1928 for determination of the percentages of homozygous plants, seeds from the upper and from the lower half of the bolls of each lot being planted separately. The resulting data are given in the lower section of Table 8.

In none of the populations resulting from pollination with different numbers of pollen grains did the subpopulations from upper and from

<sup>11</sup> The better fertilization in 1927 than in 1926 probably is accounted for by the fact that the plants used in the experiment of 1926 were somewhat stunted, while the plants used in 1927 were vigorous and healthy. The deficient fertilization in the experiments of 1926 and 1927 does not necessarily imply that there were not sufficient numbers of viable pollen grains to fertilize more of the ovules, but may have been due to isolated position on the stigmas of the relatively few grains applied. Brink (?) found that in *Cucumis* the pollen develops better when the grains are massed than when they are scattered. From this fact he inferred the secretion of mutually stimulating substances and concluded that these "are apparently products of the metabolism of the pollen tube, they are readily diffusible and are utilized more completely when the tubes are massed."

lower seeds differ significantly in the percentage of homozygous individuals, the difference in every case being smaller than its probable error. It appears that even when the total number of pollen grains (30) only slightly exceeded the number of ovules, the unlike (upland) pollen fertilized the ovules in the upper part of the ovary as readily as it fertilized the lower ovules. This finding, according with that of the experiment of 1926, indicates that there was not a more rapid growth of the tubes of the like pollen than of the unlike pollen. As the total number of pollen grains was not much greater than the number of ovules, if the tubes of the like pollen (Pima) had penetrated the ovary sooner than the tubes of the unlike pollen there should have been a higher percentage of homozygous plants in the subpopulation from upper seeds than in the subpopulation from lower seeds.

A slight but perhaps significant degree of selective fertilization was shown by the population from pollination with 30 grains, the percentage of homozygous plants being 57 per cent and the departure from 50 per cent being 3.5 times its probable error. The value of  $\chi^2$  for the departure from equal numbers of homozygous and heterozygous plants expected in the absence of selective fertilization is 11.1, indicating chances of more than 100 to 1 that the departure is significant. No selective fertilization was shown in the populations from pollination with 40, 50, and 60 grains.

The absence of evidence of a high degree of selective fertilization in the populations of this experiment and in the experiment of 1926 (Table 8) was unexpected. In the experiments of 1922 (Table 1) and in the experiment of 1925 (Table 7) in which each flower must have received many more than 60 pollen grains, selective fertilization was very pronounced, as the populations grown from seeds produced by double pollination of Pima flowers with approximately equal quantities of Pima and upland pollen contained from 67 to 91 per cent of homozygous individuals. The cause of this difference apparently lies either in the greater viability of the unlike (upland) pollen used in the experiments of 1926 and 1927 or in selective survival in favor of the heterozygotes.

The viability of the two kinds of pollen used in these later experiments was not tested directly, but all earlier tests indicated equal or nearly equal viability, and the two pollens, when applied separately to the stigmas of Pima flowers, have always produced very nearly equal degrees of fertilization. That the survival of the heterozygous plants may have been favored in the experiments of 1926 and 1927 is suggested by the fact that the soil on which these populations were grown had become heavily infested with nematodes and that at Sacaton Pima cotton is much more susceptible than upland cottons to the resulting root-knot disease. The losses from this disease have been particularly heavy in the seedling stage. In 1922 and 1925, when the earlier experiments were performed, nematode infestation was not a factor. It is a fair assumption, therefore, that more of the homozygous (Pima) than of the heterozygous (Pima  $\times$  upland) plants died from root knot in an early stage of growth, in which case unduly low proportions of homozygous (Pima) individuals would have been recorded. The appearance of a low degree of selective fertilization in favor of the like pollen in only one of the populations from the experiment of 1927 may have been due to the chance location of this population in less heavily infested soil.

The fact that in the experiments of 1926 and 1927 with mixtures of the two pollens the subpopulations from upper and lower seeds in no case differed significantly in the percentage of homozygous individuals points to the conclusion that the two pollens were substantially alike in the growth rate of their tubes. Since the excision experiment described in the preceding section also gave negative results as to difference in the rate of growth of the tubes of the two kinds of pollen when both are present on the same stigmas, the conclusion seems warranted that the tubes from such grains of the unlike pollen as escape the inhibiting action of the like pollen grow as rapidly and penetrate the ovary as soon as the tubes of the like pollen. It is inferred that the inhibiting action of the like pollen expresses itself in preventing the germination or early development of many of the unlike pollen grains.

#### SELECTIVE FERTILIZATION AS AFFECTED BY INTIMACY OF POLLEN MIXTURE AND BY CONDITION OF LIKE POLLEN

It is believed that the evidence here given has established the reality of selective fertilization in favor of the like pollen in cotton and has eliminated differential growth rate of the pollen tubes as a general cause of the phenomenon. How, then, is it to be explained? The hypothesis has been advanced by the writers (24) that a reaction takes place in the stigmatic tissue, making this tissue a less suitable medium for germination or development of the unlike pollen. The fact that the unlike pollen when present alone on the stigmas effects fertilization nearly or quite as readily as does the like pollen indicates that the inhibiting substance supposed to be produced in the stigmas develops only under a stimulus supplied by the presence of the like pollen.

If the hypothesis is well founded, the inhibiting action should be greatest when both kinds of pollen are present over the whole stigmatic surface and least when they are placed separately on different parts of the stigmatic surface. In the former case every part of the stigmatic and stylar tissues would be affected by contact with the grains and tubes of the like pollen, while in the latter case tracts of stigmatic tissue not in direct contact with the grains and tubes of the like pollen would be available for penetration by the unlike pollen. It is also essential to an understanding of the problem to know whether the assumed toxin-producing capacity of the like pollen exists only when the pollen cells of the latter are intact and viable. If so, penetration of the stigmas by the pollen tubes would be indicated as a condition of the reaction. Experiments designed to afford information on these points were carried out in 1925, 1927, and 1928.

#### ANTAGONISM PROPORTIONAL TO INTIMACY OF MIXTURE

An experiment (No. 5) was performed in 1925 to determine whether the degree of antagonism is proportional to closeness of contact of the two pollens on the stigmas. During a period of 10 days (August 7-16), 30 flowers of Pima cotton were emasculated daily and were pollinated with both Pima and Acala upland pollens. Equal numbers of flowers were pollinated in three different ways, as described below.

*Treatment A.*—Approximately equal quantities of the two pollens were applied to opposite sides of the same stigmas, so that the two pollens were separated as widely as is possible when both were present on the stigmas of the same flower.

The application in this and in treatment B was made by brushing the stigmatic surface with the staminal columns of the pollen-supplying flowers

*Treatment B*—The two pollens were applied by the method used in earlier experiments (21), Pima pollen followed by upland pollen on one-half of the number of flowers and the two pollens in the reverse order on the remaining flowers. By combining the resulting subpopulations, the result should approximate that obtained by mixing the two pollens before application to the stigmas. Since both pollens were applied to the whole surface of the same stigmas, they were in closer contact than in treatment A.

*Treatment C*—Approximately equal quantities of the two pollens were mixed together thoroughly and the mixture was applied to the stigmas. The numbers of grains of the two kinds of pollens were not counted, but the mixture used on each day of the experiment was examined before it was placed on the stigmas in order to be sure that the two kinds of pollen, readily distinguishable by the size and color of the grains, were present in approximately equal quantities.<sup>12</sup> This method assured the greatest possible intimacy of contact between the two pollens.

A difference in the viability of the two pollens might have invalidated the results. Samples of both pollens, therefore, were tested each day

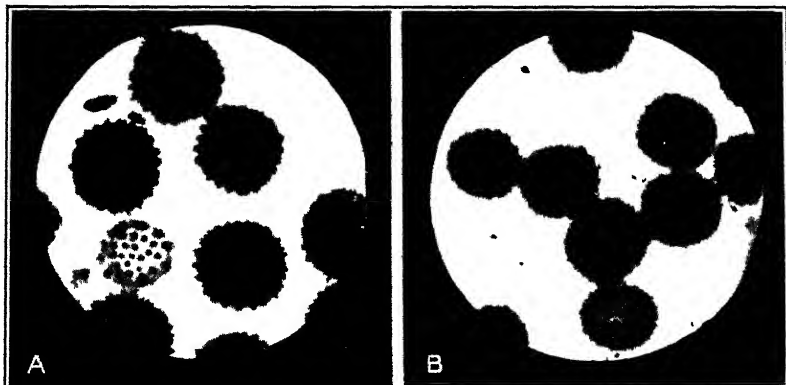


FIGURE 1—Pollen grains of Pima Egyptian cotton (A) and of Acala upland cotton (B)  $\times 160$ . The Pima pollen grains are somewhat larger and have somewhat longer spines than the upland grains. The color of the Pima pollen is deep golden yellow, and that of the upland pollen (Acala and Lone Star varieties) is pale cream. The smaller, light-colored grain in each lot of pollen is nonviable. (Photograph by A. E. Longley)

by immersion in distilled water. No appreciable difference in the proportion of grains that exploded was observed. Additional tests, in a 5 per cent sugar solution, were made at the beginning and at the end of the 10-day period of the experiment. The first test showed a decided inferiority of the Pima pollen; the percentage of defective grains ranged from 20 to 50 per cent in the several Pima flowers and was negligible in the Acala flowers. On the other hand, there was no appreciable difference between the two pollens in the promptness and completeness with which the grains of normal size exploded in the sugar solution. In the second test the estimated percentage of small, light-colored, doubtless defective grains (fig. 1) ranged from 5 to 10 per cent in the several Pima flowers and from 2 to 5 per cent in the

<sup>12</sup> Details of the procedure followed in preparing the mixture are as follows. Pima and Acala flowers were collected each morning when the anthers had begun to discharge their pollen and were brought into the laboratory. Approximately equal quantities of each pollen were shaken out on a microscope slide and mixed together thoroughly with a dissecting needle. The mixture was then transferred to gelatin capsules. The mixed pollen was applied by inverting a capsule over the stigmas and revolving it until the stigmas were well covered with pollen. The contents of each capsule served to pollinate two flowers.

several Acala flowers. The varietal difference in the proportion of such grains was therefore much less pronounced than it had been 10 days earlier. No varietal difference in the rapidity and completeness of explosion of the normal-sized grains was detected. In the pollen of both varieties, in addition to the very small grains there were a few grains of abnormally large size, which did not explode or did so only after very long immersion.

In spite of the differences shown by these tests in sugar solution, it is believed that the mixtures used in treatment C were not deficient in viable Pima pollen. The proportion of the two kinds of pollen in the mixtures was determined by inspection rather than by count and, since the deep yellow of the viable Pima grains contrasts sharply with the much paler nonviable Pima grains and the nearly colorless Acala upland grains, a marked deficiency of normal Pima pollen in the mixtures could not have escaped notice. It should be noted further that since the pollinations of this experiment were made on Pima flowers an excess of viable upland pollen in the mixtures would have increased the percentage of heterozygous plants in the resulting population and hence would have tended to nullify the effect of selective fertilization in favor of the like pollen.

The seeds obtained by the several methods of pollination were planted in 1926, and the numbers of plants grown and percentages of homozygous (Pima) individuals were determined. The results are given in Table 9.

TABLE 9—Number of plants and percentages of homozygous plants in populations from Pima flowers pollinated with both Pima Egyptian and Acala upland pollens, in different degrees of intimacy of mixture

[Experiment No 5, 1925]

Treatment	Method of applying the two pollens	Plants grown	Homozygous plants	$\chi^2$ of departures of numbers observed from numbers expected in the absence of selective fertilization
A.....	Separately, on opposite sides of the stigmas.....	Number 390	Per cent 66.6 $\pm$ 1.07	98.0
B.....	Over the whole stigmas but seriatim, Pima followed by upland pollen on half of the flowers, the sequence reversed on the other half, the populations from both sequences as 1 array *	825	77.1 $\pm$ 1.01	242.4
C.....	Mixed intimately before applying.....	978	86.4 $\pm$ .74	518.4

\* As was the case in the experiment described in an earlier paper (24, p. 332-333) the subpopulation from the like + unlike sequence of pollination gave a higher percentage of homozygous individuals (80.3  $\pm$  1.29 per cent) than the subpopulation from the sequence unlike + like (74.1  $\pm$  1.49 per cent). The difference between these percentages is 3.1 times its probable error. As the 2 subpopulations were of somewhat unequal size (431 and 394 plants, respectively) the percentage of homozygous individuals, as given for the whole population, is the average of the percentages of the 2 subpopulations.

A very high and very significant degree of selective fertilization in favor of the like pollen in all three populations is indicated by the data in Table 9. The magnitude of  $\chi^2$  of the departures of the observed numbers of homozygous and heterozygous plants from the numbers expected had there been no selective fertilization indicates, in every case, chances many more than 100 to 1 that the departure is significant. It is clear that these results are not attributable to a

If the macerated Pima pollen had had an inhibiting effect upon the capability of the unlike (upland) pollen to accomplish fertilization, then the reduction in the degree of fertilization resulting from application of the pollen extract, as compared with the fertilization of the corresponding control,<sup>14</sup> should have been greater in treatment C than in treatment A. In other words, the difference D—C should be greater than the difference B—A. Table 10 shows that this was not so in regard to percentage of bolls retained, but in mean number of seeds per boll the difference D—C is  $3.3 \pm 0.86$  seeds greater than the difference B—A. The difference between the two differences, being 3.8 times its probable error, may be regarded as significant. The results of this experiment seem to indicate that the inhibiting effect of like upon unlike pollen persists even when the viability of the former presumably has been destroyed by maceration.

In order to ascertain whether the viability of the pollen used in coating the stigmas had been destroyed completely by maceration, the seeds produced by the severally treated flowers were planted in 1928 and the populations were examined on July 19. The population representing treatment A (pollination with Pima pollen on Pima extract) comprised 218 plants, all of which were Pima, as would have been the case, of course, whether or not any of the pollen grains in the extract had remained viable. But if the Pima extract had contained viable pollen, the population representing treatment C (pollination with upland pollen on Pima extract) should have contained at least a few plants from the fertilization Pima  $\times$  Pima, whereas all of the 283 plants in this population were easily recognizable as Pima  $\times$  upland F<sub>1</sub>. It is probable that even if a few pollen grains escaped maceration by grinding in the mortar they burst promptly and lost their viability when the paste was mixed with distilled water.

TABLE 11—*Relative survival rate of plants from fertilizations Pima  $\times$  Pima (treatments A and B) and of plants from fertilizations Pima  $\times$  upland (treatments C and D), of the experiment of 1927*

Treatments	Pollination	Seed planted	Plants surviving		Nature of the plants
		Number	Number	Per cent	
A and B.....	Pima $\times$ Pima.....	1,196	592	49.5 $\pm$ 0.97	All Pima
C and D.....	Pima $\times$ upland.....	1,136	899	79.1 $\pm$ .81	All Pima $\times$ upland F <sub>1</sub>
	Difference.....			29.6 $\pm$ 1.26	

Better survival of the heterozygotes is indicated by the data given in Table 11, which shows that the combined population from flowers pollinated with unlike pollen (C and D) was much larger in proportion to the number of seeds planted than the combined population from flowers pollinated with like pollen (A and B). In earlier experiments, the heterozygotes showed no such marked superiority to the Pima plants in rate of survival,<sup>15</sup> but the soil where these plantings were

<sup>14</sup> It is interesting to note that the mean number of seeds per boll from the control flowers pollinated with upland pollen (D) is significantly greater than the number from the control flowers pollinated with Pima pollen (B), the difference being 3.9 times its probable error. Other examples of increased fertilization of Pima flowers when pollinated with upland pollen are given in Table 2.

<sup>15</sup> In the field plantings of earlier experiments, there appeared to be no superiority of the heterozygotes in this respect, as evidenced by the percentages of heterozygotes in hills containing one or two as compared with hills containing a greater number of plants (22, p. 46, 24, p. 334). In laboratory germination tests, however, there were slight differences in favor of the heterozygotes (22, Table 23). The results of a similar test, showing better germination of the heterozygous seeds, are given in Table 3 of the present paper.

A small tag, on which was written the plant number, date, and a letter indicating the treatment, was attached to each flower as soon as it was pollinated and the flower was then inclosed in a fresh bag to prevent the access of foreign pollen

The four treatments, given on each of the 10 days of the experiment to equal numbers of emasculated Pima flowers, may be summarized as follows:

- (A) Stigmas coated with Pima pollen extract, pollinated with Pima pollen
- (B) (Control) stigmas not coated, pollinated with Pima pollen
- (C) Stigmas coated with Pima pollen extract, pollinated with upland pollen
- (D) (Control) stigmas not coated, pollinated with upland pollen

A total of 100 Pima flowers received each treatment during the course of the experiment. Each of the bolls obtained, with its tag, was collected as it matured, and the number of seeds was determined. Any seed, however small, that had developed beyond the ovule stage was counted as a fertilized seed. Table 10 shows the percentages of bolls retained from flowers receiving the several treatments and the mean numbers of seeds in these bolls.

TABLE 10—Degrees of fertilization attained in Pima cotton flowers of which the stigmas had been coated with macerated Pima pollen and pollinated with Pima pollen (A) and with upland pollen (C) as compared with the control flowers of which the stigmas were not coated but were pollinated with Pima pollen (B) and with upland pollen (D)

[Experiment of 1927]

Treat- ment	Pollination of Pima	Flowers treated	Bolls re- tained	Seeds per boll
		Number	Per cent	Mean number
A.....	× Pima, on Pima extract.....	100	34 0±3 19	11 6±0 63
B.....	× Pima, control.....	100	65 0±3 22	16 2± 23
	Difference (B—A).....		31 0±4 53	4 6± 67
C.....	× upland, on Pima extract.....	100	35 0±3 22	9 6± 47
D.....	× upland, control.....	100	65 0±3 22	17 5± 24
	Difference (D—C).....		30 0±4 56	7 9± 53
	Difference (D—C)—(B—A).....		-1 0±6 43	3 3± 85

Both sets of flowers (A and C) whose stigmas had been coated with pollen extract were much less completely fertilized than the respective controls (B and D), as shown both in the lower percentage of bolls retained and in the smaller mean number of seeds per boll. This lower degree of fertilization doubtless was partly due to the fact that the coating of pollen extract prevented close contact of many of the living pollen grains with the stigmatic surface. It was observed that the pollen adhered much more readily to the untreated stigmas than to those which had been coated with the extract. The bursting of many of the grains when brought into contact with the stigmas that had been moistened by the extract probably was also a factor limiting the fertilization of the treated flowers.<sup>13</sup>

<sup>13</sup> Nearly all the bolls retained by the flowers that had received the extract were from flowers treated on the last five days of the experiment. The reason doubtless was that at first the living pollen was applied immediately after application of the pollen extract. When it was observed that practically no bolls were setting from flowers thus treated the extract was allowed to evaporate on the stigmas before the living pollen was applied. Less than one minute sufficed for this.

unlike pollen. If this had been the case, in the populations from Pima mother plants the difference D-C (upland pollen control minus upland pollen on stigmas coated with Pima pollen extract) should have been significantly greater than the difference B-A (Pima pollen control minus Pima pollen on stigmas coated with Pima pollen extract). Likewise, in the populations from upland mother plants, the difference B-E (Pima pollen control minus Pima pollen on stigmas coated with upland pollen extract) should have been significantly greater than the difference D-G (upland pollen control minus upland pollen on stigmas coated with upland pollen extract).

TABLE 12 — Number of flowers treated, percentage of bolls retained, and mean number of seeds per boll from emasculated flowers of *Pima* Egyptian and of *Acala* upland cotton, some of which had been pollinated with *Pima* pollen and some with *Acala* pollen, with and without previous application to the stigmas of macerated pollen (pollen extract) of each type of cotton

[Experiment of 1928]

PIMA AS MOTHER PLANTS

Treatment	Pollination	Flowers treated	Bolls retained	Seeds per boll *
		Number	Per cent	Mean number
A .....	X Pima, on Pima extract .....	50	74 0±4 18	9 3±0 75
B .....	X Pima, control .....	101	97 0±1 15	18 0± 21
	Difference (B-A) .....		23 0±4 34	8 7± 78
C .....	X upland, on Pima extract .....	51	80 4±3 75	9 6± 71
D .....	X upland, control .....	100	93 0±1 72	18 2± 23
	Difference (D-C) .....		12 6±4 13	8 6± 75
	Difference (D-C)-(B-A) .....		-10 4±6 00	- 1±1 08
E .....	X Pima, on upland extract .....	50	72 0±4 28	9 5± 69
B .....	X Pima, control .....	101	97 0±1 15	18 0± 21
	Difference (B-E) .....		25 0±4 44	8 5± 72
G .....	X upland, on upland extract .....	50	68 0±4 45	8 2± 59
D .....	X upland, control .....	100	93 0±1 72	18 2± 23
	Difference (D-G) .....		25 0±4 77	10 0± 63
	Difference (B-E)-(D-G) .....		0 ±6 52	-1.5± 96

UPLAND AS MOTHER PLANTS

A .....	X Pima, on Pima extract .....	50	44 0±4 74	11 6±1 05
B .....	X Pima, control .....	50	96 0±1 87	31 1± 51
	Difference (B-A) .....		52 0±5 10	19 5±1 17
C .....	X upland, on Pima extract .....	50	36 0±4 58	14 4±1 43
D .....	X upland, control .....	50	98 0±1 33	33 0± 25
	Difference (D-C) .....		62 0±4 77	18 6±1 46
	Difference (D-C)-(B-A) .....		10 0±6 99	- 9±1 86
E .....	X Pima, on upland extract .....	51	41 2±4 65	10 8± 92
B .....	X Pima, control .....	50	96 0±1 87	31 1± 51
	Difference (B-E) .....		54 8±5 02	20 3±1 05
G .....	X upland, on upland extract .....	50	28 0±4 28	14.9±1 57
D .....	X upland, control .....	50	98 0±1 33	33 0± 25
	Difference (D-G) .....		70 0±4 49	18 1±1 59
	Difference (B-E)-(D-G) .....		-15 2±6 74	2 2±1 91

\* Probable errors increased by using Pearson's correction for the standard deviation when the number is small.

made in 1928 was heavily infested with nematodes, and it has been observed at Sacaton that the mortality due to these organisms is much heavier in Pima than in upland cotton or in Pima  $\times$  upland  $F_1$ .

#### EXPERIMENT OF 1928

The results of the experiment in 1927 indicated that Pima pollen of which the viability has been destroyed by maceration may inhibit the germination or subsequent development of some of the upland pollen grains on the stigmas of the Pima flowers. The observed effect was slight, however, and it was thought advisable to repeat the experiment on a larger scale. This was done in 1928. The number of treatments was increased by using macerated pollen of both Pima and Acala upland and by applying it to flowers of both varieties. The several lots of emasculated flowers of each variety, therefore, were treated as follows:

- (A) Stigmas coated with Pima pollen extract, pollinated with Pima pollen.
- (B) (Control) stigmas not coated, pollinated with Pima pollen.
- (C) Stigmas coated with Pima pollen extract, pollinated with upland pollen.
- (D) (Control) stigmas not coated, pollinated with upland pollen.
- (E) Stigmas coated with upland pollen extract, pollinated with Pima pollen.
- (G) Stigmas coated with upland pollen extract, pollinated with upland pollen.

The first four treatments are the same as treatments A, B, C, and D in the experiment of 1927, while treatments E and G amplify the experiment so as to test the effects of macerated upland pollen also. Treatment B (Pima pollination) served as the control for treatments A and E, and treatment D (upland pollination) served as the control for treatments C and G.

The technic was the same as that of the 1927 experiment, except in two minor details. From the plants selected for treatment all bolls that had set before the beginning of the experiment were removed at the outset, and thereafter all flowers not treated were removed daily. This was done because of Eaton's discovery that removal of many of the flowers and bolls reduces the rate of shedding of those left on the plants (10). It was found that the soft, almost flaccid involucre of the upland flowers were much in the way when emasculating and pollinating, a difficulty not experienced in handling the Pima flowers because of the crisper, more rigid texture of the involucre in this type. For this reason the upper portion of the upland involucre was cut away when emasculating the flower bud.

The percentage of bolls retained and the mean number of seeds in these bolls were computed separately for each of the six treatments in each population. The statistical constants thus obtained are given in Table 12. The percentage of bolls retained and the mean number of seeds per boll were much lower from flowers treated with pollen extract (treatments A, C, E, and G) than from the control flowers (treatments B and D). The reason for this difference was given in describing the experiment of 1927.

Table 12 shows also the difference in percentage of bolls retained and in mean number of seeds per boll between each lot from flowers treated with macerated pollen and the lot from the corresponding control flowers. Comparison of the differences between these differences, likewise given in Table 12, should determine whether the macerated pollen had reduced the degree of fertilization effected by the

in 1929 and the populations thus obtained were classified, with the results given in Table 13. Except for the two cases noted in the footnote to the table, of the occurrence of homozygous (upland) plants in populations which should have contained only heterozygous (upland  $\times$  Pima  $F_1$ ) plants, the classification indicates complete destruction of the viability of the macerated pollen. The exceptions are attributable to volunteering from seeds left in the soil from the preceding crop. The fact that no heterozygous plants were found in populations that should have been all homozygous warrants the conclusion that the maceration had been effective.

#### COMPARISON OF THE TWO EXPERIMENTS WITH MACERATED POLLEN

The experiment of 1927, in which only Pima mother plants were used (see Table 10) showed that in mean number of seeds per boll the difference  $D - C$  (upland pollen control minus upland pollen on Pima stigmas coated with Pima pollen extract) was greater than the difference  $B - A$  (Pima pollen control minus Pima pollen on Pima stigmas coated with Pima pollen extract), the difference between these differences ( $D - C$  minus  $B - A$ ) being positive in sign and nearly four times its probable error. This result indicated that the treatment with macerated Pima pollen depressed the fertilization of flowers pollinated with the unlike (upland) pollen more than it depressed the fertilization of flowers pollinated with the like (Pima) pollen.

The more comprehensive experiment of 1928, in which plants of both Pima and upland cotton were used as mothers and the effects of extracts of both kinds of pollen were tested, showed, on the contrary, that treatment with like pollen in a macerated condition caused no significant differences in the relative fertilization of flowers of either type whether the pollen supplied in a living state was of the same or of the other type. The presence of the macerated pollen on the stigmas resulted in greatly reduced fertilization, but the action seems to have been purely mechanical, since the reduction was no greater when the living pollen was of the other type than when it was of the same type as the macerated pollen.

The greater weight of evidence, therefore, favors the conclusion that the hypothetical alteration in the stigmatic tissue, unfavorable to the unlike pollen and attributed to action of the like pollen when both kinds are present on the stigmas, takes place only when the like pollen is viable. If such a reaction in the stigmatic tissue is the true explanation of selective fertilization as observed in cotton, presumably the reaction can take place only after the tubes of the like pollen have penetrated the stigmas, a condition that is removed by maceration of the like pollen.

#### POLLEN ANTAGONISM IN RELATION TO CONSANGUINITY

Jones (21, p. 75) found that in maize the degree of selective fertilization in favor of the like pollen is positively and rather highly correlated with the degree of heterosis indicated by the weight of the heterozygous seeds. The coefficient of correlation obtained was  $0.50 \pm 0.09$ . In other words, assuming that the greater the degree of heterosis the more remote is the relationship of the two forms that are crossed, it follows that selective fertilization tends to be inversely

Since the hypothesis assumes an inhibiting effect of the like pollen upon the unlike pollen but no such effect of the unlike pollen upon the like pollen, there should have been no significant difference between the pollinations with like and with unlike pollen in the degree to which the fertilization of the flowers of either variety was reduced by the application of macerated pollen of the other variety. In other words, in the populations from Pima mother plants, the difference B—E (Pima pollen control minus Pima pollen on stigmas coated with upland pollen extract) should not have differed significantly from the difference D—G (upland pollen control minus upland pollen on stigmas coated with upland pollen extract). Likewise, in the populations from upland mother plants, the difference D—C (upland pollen control minus upland pollen on stigmas coated with Pima pollen extract) should not have differed significantly from the difference B—A (Pima pollen control minus Pima pollen on stigmas coated with Pima pollen extract).

It is evident from the data in Table 12 that none of these differences between differences, in either percentage of bolls retained or mean number of seeds per boll, is significant with respect to its probable error. In this experiment, therefore, there was no indication that either pollen, when macerated and applied to the stigmas of either variety, exerted an inhibiting action upon the development of the other kind of pollen, except for the purely mechanical effects of the presence of the pollen extract, as noted above.

TABLE 13.—*Classification of populations from the several treatments of flowers on plants of Pima and of Acala upland cotton*

[Experiment of 1925]

SEEDS FROM PIMA MOTHER PLANTS

Treatment	Pollination	Number of plants grown			Expectation if maceration was completely effective (all plants)
		Total	Homozygous	Heterozygous (F <sup>1</sup> )	
A.....	× Pima, on Pima extract.....	20	20	0	Homozygous.
B.....	× Pima, control.....	117	117	0	Do
C.....	× upland, on Pima extract.....	60	0	60	Heterozygous
D.....	× upland, control.....	449	0	449	Do
E.....	× Pima, on upland extract.....	33	33	0	Homozygous
G.....	× upland, on upland extract.....	48	0	48	Heterozygous

SEEDS FROM UPLAND MOTHER PLANTS

A.....	× Pima, on Pima extract.....	149	* 35	114	Heterozygous
B.....	× Pima, control.....	188	0	188	Do
C.....	× upland, on Pima extract.....	109	109	0	Homozygous
D.....	× upland, control.....	111	111	0	Do
E.....	× Pima, on upland extract.....	124	* 18	106	Heterozygous
G.....	× upland, on upland extract.....	33	33	0	Homozygous

\* The occurrence of a few homozygous (upland) plants in these 2 populations, where none was expected, is attributable to the fact that the land had been in upland cotton the year before and many plants volunteered from seeds left in the soil.

In order to ascertain whether any error could have arisen through failure to destroy the viability of all the pollen grains in the macerated pollen, the seeds resulting from the several treatments were planted

population was considerably in excess of the 50 per cent expected had there been no selective fertilization. The departure amounts to 10.4 per cent and is four times its probable error. The more advantageous position of the pollen applied first on the stigmas may partly account for this excess (24, p. 332, 333), but if this had been the only factor there should have been a corresponding excess of heterozygous plants in the population from flowers that received the unlike pollen first. In fact, however, there was a small but not significant excess of homozygous plants in the latter population.<sup>16</sup>

TABLE 14.—Number of plants and percentage of homozygous plants in the several populations resulting from pollination of flowers of a homozygous weak-spotted family of *Prima* cotton with pollen of both the weak-spotted family and of a homozygous full-spotted family of the same variety

[Experiment of 1924]

Population based on—	Plants grown	Homozy- gous plants
	Number	Per cent
Sequence of pollination		
Like + unlike (all seeds).....	321	60.4 ± 1.84
Unlike + like (all seeds).....	234	53.8 ± 2.20
As one array (all seeds).....	555	57.1 ± 1.42
Position of seeds in boll		
Upper seeds (both sequences).....	245	62.4 ± 2.09
Lower seeds (both sequences).....	310	53.9 ± 1.91

\* Because of the difference in size of the subpopulations from the like + unlike sequence of pollination (all seeds) and the unlike + like sequence (all seeds) the percentage for the whole population (555 individuals) was not computed directly from the total number of plants and of homozygous individuals but was taken as the average of the percentages of the subpopulations representing the 2 sequences of pollination.

The average of the percentages of homozygous individuals in the two subpopulations (from like + unlike and from unlike + like pollinations) should approximate the result that would have been obtained if equal quantities of the two kinds of pollen had been mixed together before application to the stigmas. The average, given in Table 14 as the percentage of the whole population, shows an excess of 7.1 per cent of homozygous plants, and the departure from the  $50.0 \pm 1.42$  per cent expected had there been no selective fertilization in favor of the like pollen is 3.5 times its probable error. The departures of the observed numbers of homozygotes and heterozygotes from the equal numbers expected if there had been no selection among the pollen grains give a value for  $\chi^2$  of 11.2, indicating chances of more than 100 to 1 that the departures are significant.

The question whether the tubes of the like pollen grew faster than the tubes of the unlike pollen is also of interest. If such were the case, there should have been a higher percentage of homozygous individuals in the population from seeds in the upper part of the boll than in the population from seeds in the lower part of the boll. The data given in the lower section of Table 14 indicate that this was the case, the percentage of homozygous individuals being  $85 \pm 2.83$  per cent greater from the upper than from the lower seeds. The difference is only three times its probable error, hence doubtfully significant,

<sup>16</sup> The departure in the subpopulation from unlike + like pollination from the expectation (60.4 per cent heterozygotes), if sequence of pollination had been the only factor and had had the same effect as in the like + unlike population, is 14.1 (60.4 per cent of 234) - 108 (the actual number heterozygous) = 33 ± 5.05. The probable error of this departure was computed as

$0.6745 \times \sqrt{\frac{(0.604) \times 1 - (0.396) \times n}{n(234)}}$ .

proportional to consanguinity As Jones expressed it, "the somewhat surprising situation exists that in proportion as the cross-fertilization benefits the immediate progeny in its development the less effective is that pollen in accomplishing the union "

In the case of cotton, the writers obtained evidence of a high degree of selective fertilization when two kinds of pollen, each representing a distinct species of *Gossypium*, were applied to the stigmas of the same flower of one of these species. To ascertain whether the situation is similar to that in maize, experiments involving more nearly related forms were undertaken Two families of Pima cotton that differ in a single Mendelian character,  $1 e$ , full and very weak development of the spot at the base of the petal, afforded suitable material for testing this point, since both parental families were homozygous and the heterozygotes of crosses between them could be distinguished easily from the recessive (weak-spotted) parental form.

#### EXPERIMENT OF 1924

In 1924, plants of the weak-spotted Pima progeny 3-27-29 were grown at Sacaton adjacent to plants of the full-spotted Pima progeny 13-17-10. Flowers of the former were emasculated and pollinated with both kinds of pollen, taken at random from the several individuals in the respective progeny. The technic was the same as in several of the experiments with Pima and upland cotton,  $1 e$ , one half of the flowers were pollinated first with like and then with unlike pollen (like + unlike) and the other half were pollinated first with unlike and then with like pollen (unlike + like)

Equal numbers of flowers received each treatment on each day, and practically the same number of seeds was obtained from each treatment The bolls that matured from each lot of flowers were halved and the seeds from the upper and from the lower half were kept separate, giving four subpopulations that were grown in 1925 These subpopulations represented: Like + unlike pollination, upper seeds; like + unlike pollination, lower seeds, unlike + like pollination, upper seeds, and unlike + like pollination, lower seeds Four seeds were planted to the hill, and no thinning was done Although equal numbers of seeds were planted, the stand varied so greatly in different parts of the plot that the several subpopulations were of very unequal size

As the plants flowered they were classified To guard against errors in classification, three flowers on each plant were graded as to the degree of spotting, and the average of the grades of the three flowers was determined The two classes of plants were easily distinguished, 3 0 representing the maximum average grade in the weak-spotted class and 5 5 the minimum average grade in the strongly spotted class. It may safely be concluded that the former were homozygous, representing the fertilization like  $\times$  like, and that the latter were heterozygous, representing the fertilization like  $\times$  unlike

Table 14 shows the number of plants and percentage of homozygous individuals in the subpopulations as combined in two pairs representing, respectively, the two sequences of pollination and the two positions of the seeds.

The upper section of Table 14 shows that when the like pollen was applied first the percentage of homozygous individuals in the resulting

harmony with those obtained by Jones (21) in maize, indicating that selective fertilization diminishes with increasing consanguinity of the two forms.

#### HYPOTHESIS OF POLLEN ANTAGONISM

It has been demonstrated by the writers that when pollens of both Pima and upland cotton are applied in approximately equal quantities to the stigmas of emasculated flowers of either type the resulting progeny shows a marked preponderance of plants from fertilization by like pollen (Pima  $\times$  Pima or upland  $\times$  upland). In other words, unlike pollen is at a disadvantage in fertilizing the flowers when like pollen also is present on the stigmas, as shown in Tables 1, 7, and 9.

If there were any lack of compatibility between the two types of cotton, these results would require no further explanation, but there is abundant evidence, summarized in Table 2, of nearly perfect compatibility when either pollen is applied alone to stigmas of the other type. This nearly or quite complete mutual compatibility of Pima and upland cotton is surprising in view of the fact that they belong to very distinct species, representing each of the main groups of cultivated American cottons. Pima (*Gossypium barbadense*) is of the South American group and upland (*G. hirsutum*) is of the Mexican group (23, p. 207, 208). These types differ in many morphological characters and also in such physiological characters as rate of boll shedding (25, p. 652, 653) and selective absorption of certain components of the soil solution (15, 16).

A pronounced difference in the viability of the pollens also would account for the observed facts, but repeated tests *in vitro* have given no evidence of differences sufficient to account for the observed inequalities in the proportion of homozygous and heterozygous plants shown by the populations from mixed pollinations.

Differential survival, after fertilization, in favor of the homozygotes would have the same effect as selective fertilization in favor of the like pollen, but in all cases where differences have been observed in the rate of survival, during or after germination, the advantage has been with the heterozygotes. Such differential survival as has occurred in the experiments, therefore, has been of a nature to obscure rather than to magnify the effect of selective fertilization in favor of the like pollen.

It is also possible that there may have been selective survival among the zygotes immediately after fertilization, resulting in the death at a very early stage of a disproportional number of zygotes from like  $\times$  unlike unions. The nearly perfect compatibility of the two cottons and the greater vigor of the heterozygotes at and after the stage of germination make this improbable, but the possibility should not be ignored. Comparison of the mean number of seeds with the mean number of ovules, assuming that all undeveloped ovules represent unsuccessful heterozygous unions, should indicate whether this assumption may account for the observed preponderance of homozygous plants in the adult population. Computation on this basis, in five populations in which there was a marked indication of selective fertilization, increased the percentage of possible heterozygous individuals from 32.4 to 47.0, from 17.2 to 35.3, from 11.8 to 23.7, from 9.3 to 20.0, and from 12.8 to 21.4. In all but one of these populations the

but the fact that it is of nearly the same magnitude as the excess in percentage of homozygous individuals in the whole population suggests that the small degree of selective fertilization shown in this experiment may have been due to the more rapid growth of the tubes of the like pollen. It is possible, therefore, that these families of Pima cotton differ genetically in their determiners for rate of pollen-tube growth.

#### EXPERIMENT OF 1925

The experiment of 1924 was repeated the following year on material from the same homozygous Pima families, weak-spotted and full-spotted. The pollinations were made in 1925, and the resulting seeds were planted in 1926. The technic was in all respects similar to that of the first experiment except that the upper and lower seeds were not separated. The results are given in Table 15.

TABLE 15—*Number of plants and percentage of homozygous plants in the populations resulting from pollination of flowers of a homozygous weak-spotted family of Pima cotton with pollen of both the weak-spotted family and a homozygous full-spotted family of the same variety*

[Experiment of 1925]

Sequence of pollination	Plants grown	Homozygous plants
	<i>Number</i>	<i>Per cent</i>
Like + unlike.....	507	54.8 $\pm$ 1.49
Unlike + like.....	524	36.1 $\pm$ 1.41
As one array.....	1,031	* 45.45 $\pm$ 1.05

\* Average of the percentages of the 2 populations

In this experiment the sequence in which the two pollens were applied made a much larger and much more significant difference between the resulting percentages of homozygous individuals than was the case in the experiment of 1924, indicating that the stigmas in the later experiment may have been more densely covered with the pollen applied first in the sequence unlike + like than in the earlier experiment. The average of the percentages of the two subpopulations in the present experiment differs from that of the earlier experiment in showing a deficit (45.45  $\pm$  1.05) instead of an excess (57.1  $\pm$  1.42) of homozygous individuals. (See Tables 14 and 15.) The departure from the 50 per cent expected if there had been no selective fertilization is only three times its probable error, but the deviations from equal numbers of homozygous and heterozygous individuals give a value for  $\chi^2$  of 8.75, indicating chances of more than 100 to 1 that the deviations were significant.

The two experiments, therefore, although alike in the technic and material employed, gave opposite results. A small degree of selective fertilization is indicated by both experiments, but in the first experiment the selection was in favor of the like pollen and in the second experiment it was in favor of the unlike pollen. As the matter stands at present, it may be concluded that these two families of the same species and variety of cotton are so closely related that there is very little, if any, antagonism between their pollens when present together on the same stigmatic surface. So far as they go, the results are in

of the pollens, or selective survival at any stage after union of the male and female gamete, and (2) that differential growth rate of the pollen tubes is not the explanation, such of the tubes of the unlike pollen as develop being able to penetrate the ovary as rapidly and to accomplish fertilization as readily as the tubes of the like pollen. It would seem that the phenomenon is essentially different from selective fertilization in *Zea*, *Oenothera*, and *Melandrium*, which is attributed to genetic differences in the rate of pollen-tube growth. The writers, therefore, have chosen the term "pollen antagonism" to designate the supposed cause of selective fertilization as observed in cotton.

The only hypothesis of pollen antagonism that seems applicable was stated in an earlier publication, as follows (24, p 339):

\* \* \* the presence of like pollen in some way prevents the germination or subsequent development of many of the unlike pollen grains when both kinds are present on the stigmas. That the inhibiting factor does not reside in the stigmas themselves when like pollen is absent seems clear from the fact that when applied separately the unlike pollen is not inferior to the like pollen in rapidity of development and ability to effect fertilization. It is conceivable, however, that the presence of pollen of the same type may induce a physiological reaction in the stigmas which makes them a relatively unfavorable medium for the germination or growth of pollen of a different type. The further assumption must be made that, in spite of this unfavorable condition, some of the unlike pollen grains are able to accomplish fertilization, possibly because they are more resistant, possibly because they happen to be so placed as to avoid the tracts of stigmatic tissue affected by contact with the like pollen.

The results of experiments described in the present paper are believed to support this hypothesis. It is shown (Table 9) that the degree of selective action is proportional to the intimacy of mixture of the two pollens. The excess of homozygous individuals in the resulting population was least when the two pollens were deposited separately on opposite sides of the stigmas and greatest when an intimate mixture of the two pollens was applied to the whole stigmatic surface. This finding favors the assumption that the development of the unlike pollen tubes is less hindered in tracts of stigmatic and stylar tissue not immediately in contact with the tubes of the like pollen. Evidence also was obtained (Table 12) that the ability of the like pollen to inhibit the development of the unlike pollen is lost when the viability of the former is destroyed by maceration, from which it is inferred that the tubes of the like pollen must penetrate the stigmas in order to produce the inhibiting reaction.

A reaction of some kind within the stigmatic tissue is suggested by the fact that the results of the pollinations with mixed pollen in the earlier experiments (summarized in Table 1) showed the selective fertilization in favor of the like pollen to be of the same order of magnitude, whether the two pollens were applied to Pima or to upland cotton flowers. To account for the phenomenon on the basis of a direct toxic action of the one kind of pollen on the other would require the improbable assumption that the Pima pollen is toxic to the upland pollen only when the two kinds happen to be present on Pima stigmas and that upland pollen is toxic to Pima pollen only on the upland stigmas. The fact that there was selective fertilization in favor of the like pollen resulting from treatment A of the experiment of 1925 (Table 9) in which the two kinds of pollen grains, although present on

deficit of heterozygous plants is still far too great to warrant the assumption that selective survival rather than selective fertilization is the explanation<sup>17</sup>

The evidence seems conclusive that the observed facts may be interpreted only on the basis of selection between the two kinds of pollen grains. Difference in the rate of growth of the pollen tubes is the explanation that suggested itself first.

It is obvious that if two kinds of pollen differ consistently in this respect, the faster-growing pollen will have the advantage in fertilizing the ovules when both kinds are present on the stigma of the same flower. Evidence obtained by Jones and others and reviewed by Jones (21, p 6-34) indicates that in maize, *Oenothera*, *Melandrium*, *Datura*, and other plants the two kinds of male gametes produced by a heterozygous individual may differ in the rapidity with which they effect contact with the female gametes, and that as a result there is selective fertilization and a consequent distortion of expected Mendelian ratios. In these cases it appears that the difference is of a genetic nature and that frequently the gene determining the rate of growth of the tube is linked with a gene for some visible character. As expressed by Brieger (3, p 187), "Mendelian factors exist which produce a selection among the gametes of a plant heterozygous for these factors, and both the mating of like with like or of unlike with unlike may be favored." Jones also found evidence (21, p 72-73) that the pronounced selective fertilization in favor of the like pollen, observed by him in maize when a mixture of two kinds of pollen was applied to the same stigmas, was caused, at least in part, by the more rapid growth of the tubes of the like pollen.

Is selective fertilization in cotton to be explained on this basis? The evidence obtained by pollination of flowers of Pima and of upland cotton with pollen of both types was negative. When the pollens were applied separately and the styles and stigmas were excised at successive intervals, consistently greater fertilization, at a relatively short interval after pollination, was not effected by the like pollen as compared with the unlike. When the pollens were applied mixed there was not a greater proportion of homozygous plants in the population from flowers of which the styles had been excised comparatively soon after pollination than in the population from flowers in which, by postponing or omitting the excision, a longer period had been afforded for penetration of the ovary by the pollen tubes. Finally, comparison of the populations from seeds in the upper and in the lower part of the capsules obtained by pollinating flowers with mixed pollen did not show a higher percentage of homozygous plants in the population from upper seeds, as should have been the case, according to the results of Correns's experiments with *Melandrium* (8), if there had been more rapid growth of the tubes of the like pollen.

It may be concluded, therefore, (1) that selective fertilization between Pima and upland cottons really occurs, the excess of homozygous individuals in the populations from mixed pollination not being attributable to lack of mutual compatibility, different viability

<sup>17</sup> Moreover, the assumption that all undeveloped ovules represent unsuccessful heterozygous unions is unwarranted, since, even when the flowers are pollinated with like pollen only and in abundant quantity, some of the ovules always fail to develop. The data in Table 2 indicate that when Pima flowers were fertilized with Pima pollen the mean number of seeds in the resulting bolls in no case exceeded 18, whereas the mean number of ovules in the Pima ovary is approximately 21.5.

and diffusing into adjacent tissues of the mother plant, produces certain alterations in the latter. Metaxenia, discovered in the date palm, has been found by one of the writers (17) to occur in cotton also<sup>21</sup>

Pollen antagonism seems more analogous to anaphylactic than to hormone reactions in animal bodies. The action of the like pollen may be compared to that of an antigen, stimulating the production of antibodies in the tissue into which it is introduced. The analogy is far from perfect, because, in the case under consideration, the pollen presumably acting in the manner of an antigen is genetically like the body in which the reaction is supposed to take place, and the resulting "antibody" is supposed to attack the foreign pollen.<sup>22</sup>

There is as yet little evidence of the occurrence in plants of substances comparable to hormones and antibodies, but the extensive occurrence and great importance in the animal kingdom of hormone effects and of anaphylactic reactions makes it reasonable to suppose that analogous substances and reactions occur in the vegetable kingdom.<sup>23</sup> The discovery of such phenomena as metaxenia and pollen antagonism suggests that biochemical research in this field may prove fruitful

#### SUMMARY

Emasculated flowers of Pima and of upland cotton, pollinated with approximately equal quantities of pollen of both types, have shown a marked degree of selective fertilization; the resulting populations have contained a much higher percentage of homozygous than of heterozygous plants

Application of either pollen separately showed that these cottons are highly compatible, as fertilization of the flowers of either Pima or upland cotton has been effected almost or quite as readily by the unlike as by the like pollen.

So far as could be determined by observation and by tests in media not suitable for normal germination, there were no differences in the viability of the two pollens that could account for the selective action observed.

No evidence was obtained of selective survival at any stage after formation of the zygote that would explain the preponderance of homozygous plants in the populations. Selective survival undoubtedly occurred in some of the experiments, owing to infestation of the soil with nematodes, which are known to cause much greater mortality among the Pima plants than among the Pima × upland F<sub>1</sub> plants. In all such cases survival of the heterozygotes was favored, so this

<sup>21</sup> The presence of hormonelike substances in plants had been suggested previously by Haberlandt (13, p. 41), who considered that the results of his experiments on cell division in wound tissues demonstrated the existence of what he terms "division hormones," supposed to be secreted by cells of the leptome tissue, and "wound hormones," secreted by the injured cells themselves. He did not ascertain the chemical nature of the assumed substances but suggested that they may be amines.

<sup>22</sup> Wells (34, p. 703) states that "as a general rule, the more closely related the animal furnishing the antibodies is to the one furnishing the antigen the less antigenic activity or antibody response will be obtained." He adds, however, "Some proteins \* \* \* may be so foreign to the blood stream and the active tissues of the body that they incite antibody formation when introduced into the blood stream of even the same animal from which they came."

<sup>23</sup> Antibody production has been suggested as an explanation of immunity to diseases in resistant races of plants, but positive evidence apparently is lacking. Kostoff (26, p. 73) summarizes as follows the results of experiments in which various species of Solanaceae were grafted one on another: "\* \* \* mutual induction of antibodies in scion and in stock was found. The acquired immunity in such plants was tested by precipitin reactions. The induced antibodies were specific in certain species." Silberschmidt (32) criticizes certain details of the methods used by Kostoff and concludes that the results of his own experiments do not indicate the occurrence of "acquired" precipitins in plants to the extent that antibodies occur in animals.

the same stigmas, were not in contact, is further evidence against a direct toxic action of the one kind of pollen on the other<sup>18</sup>

In seeking an explanation of a very different phenomenon, self-sterility as observed in *Nicotiana*, East and Park (9) inferred the occurrence of a chemical reaction in the pistil induced by pollen, in this case by the unlike pollen. They wrote (9, p 363-364).

These results appear to us to show that the pollen tubes in a selfed pistil are not inhibited in their growth by substances secreted in that pistil, but rather that a substance or substances are secreted in the pistil after a compatible cross which accelerate growth, and that the direct cause of this secretion is a catalyzer which the pollen-tube nucleus is able to produce because the zygotic constitution of the plant producing it is different in certain particular hereditary factors from that of the plant on which it is placed \* \* \*. The action must be local, because the presence of compatible pollen tubes does not accelerate the growth of self pollen tubes

That the assumed reaction in the pistil of the cotton plant also must be local is indicated by the fact that the selective effect was least when the two pollens were deposited separately on opposite sides of the stigmas

Yasuda (35) sought to determine whether the "Linienstoffe," as Correns termed the substances assumed by him to control compatibility, are secreted in the style or in the ovary. He performed an ingenious experiment, involving two self-incompatible strains of *Petunia*, in which styles of each strain were grafted on ovaries of each strain, giving four combinations, with intact pistils of each strain as controls. It was found that the growth rate of the tubes of both kinds of pollen was determined by the identity of the ovary and not of the style in the artificial systems. Yasuda concludes that the Linienstoffe are secreted by the ovary and diffuse thence into the style<sup>19</sup>

No evidence as to the nature of the inhibiting substance supposed to be produced in the pistil of the cotton plant is now available. Biochemical tests of extreme delicacy probably would be required to detect it. It is conceivable that the substance acts so as to render ineffective one or more of the enzymes present in the pollen grains and requisite for penetration of the tissues of the pistil and for utilization of the reserve food stored in them<sup>20</sup>

A hypothesis advanced by Swingle (33) in explanation of metaxenism, or direct effect of pollen on tissues of the mother plant, is of interest in this connection. Swingle suggests that a hormonelike substance, secreted after fertilization by the embryo or the endosperm

<sup>18</sup> The phenomenon, therefore, differs from that observed in animals by Godlewski (11), who found that eggs of a sea-urchin could be fertilized by sperm of a worm, but if the sperms of both animals were mixed together, both lost their ability to fertilize the sea-urchin eggs. Results similar to Godlewski's, from experiments in vitro with plant pollen, are reported by O'Connor (27), who found that development of the tubes of one kind of pollen was checked or inhibited in the presence either of pollen or of extracts of the stigmas and other tissues of a plant belonging to another species, genus, or family. He concluded (27, p 480) that the inhibiting substances probably are amino compounds and that "in angiosperms, each species contains within each cell substances which are toxic to foreign pollen."

<sup>19</sup> It would be difficult to explain pollen antagonism as observed in cotton on the basis of an ovarian secretion unless the selection between the two kinds of pollen becomes operative only after their tubes have entered the ovary. Otherwise it would be necessary to assume that a stimulus initiated by the tubes of the like pollen as they penetrate the stigmas is transmitted downward to the ovary, causing production of a secretion which, diffusing up through the style, acts unfavorably upon the development of the tubes of the unlike pollen.

<sup>20</sup> Paton (28) investigated the pollen of 18 species of plants and detected 10 different enzymes 5, of which, including pectinase, were present in all the pollens. Green (12, p 409) found that "the style itself contains enzymes to assist in preparing the reserve materials for absorption by the pollen tube, while the latter excretes the same ferments during its progress down the conducting tissue." The same investigator also discovered that "when the pollen grain has lost the power of germinating, the quantity of diastase has materially decreased." The complexity of the chemistry and physiology of pollen has been brought out in studies by Brink and by other investigators whose work he reviews (4, 5, 6, 7).

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factor tended to nullify rather than to accentuate the appearance of selective fertilization

Comparisons of the degrees of fertilization effected when the styles were excised at shorter and at longer intervals after pollination and of populations grown from seeds in the upper and in the lower half of the boll gave no consistent evidence of a differential rate of growth of the tubes of the like and of the unlike pollen. This factor, apparently responsible for selective fertilization as observed in *Zea*, *Oenothera*, and other plants, does not account for the situation met with in *Gossypium*

The only explanation that seems tenable is that the presence of the like pollen induces a reaction in the stigmatic tissues of such nature as to render them less suitable for the development of the unlike pollen. Apparently the effect is extremely local or else individual pollen grains differ greatly in their ability to withstand the unfavorable condition, since in all the experiments with pollen mixtures some of the ovules were fertilized by the unlike pollen

Evidence of the localization of the reaction was afforded by an experiment in which the like and unlike pollens were (1) deposited separately on opposite sides of the same stigmas; (2) mixed, but not intimately, and applied to the whole surface of the stigma; and (3) mixed intimately and applied to the whole surface. The percentage of homozygous plants was least in the population from treatment 1 and greatest in the population from treatment 3.

Experiments in which the viability of the like pollen was destroyed by maceration before it was applied to the stigmas gave conflicting results, but the weight of the evidence favors the conclusion that the inhibiting effect upon the unlike pollen takes place only when the like pollen is intact and viable. Therefore, penetration of the stigmas by the tubes of the like pollen seems requisite to the setting up of the reaction. This supports the assumption that the inhibiting substance is produced in the stigmatic or stylar tissue in response to a stimulus supplied by the tubes of the like pollen.

If the hypothesis is well founded, the phenomenon observed in cotton is of a chemical or physiological nature. The term "pollen antagonism" is suggested in order to distinguish it from the selective fertilization observed in other plants and attributed to differential growth rate of the pollen tubes, conditioned by specific genes determining the rates of growth

Little or no selective fertilization was observed between more nearly related forms, these being two families of Pima cotton differing only in a simple Mendelian character. The evidence is too scanty, however, to warrant the conclusion that the degree of pollen antagonism in cotton is definitely related to the degree of consanguinity, as Jones found to be the case with selective fertilization in maize.

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# LIGHT INTENSITY IN RELATION TO PLANT GROWTH IN A VIRGIN NORWAY PINE FOREST<sup>1</sup>

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## INTRODUCTION

One of the vital problems confronting foresters in the United States is that of securing a vigorous stand of young trees to replace those removed in logging. Any cutting necessarily changes the conditions for plant growth in the forest. The forester is concerned with the type of cutting which will prove most favorable for the establishment and growth of a new stand.

The virgin forest offers an ideal place to study the effects of various factors on forest reproduction, because in it can be found trees of various ages, openings of different sizes, and many densities of upper canopies, in addition, an ample seed supply is usually present. It should be possible, therefore, from a careful study of conditions in virgin stands, to gain much information on the requirements of the young growth and on what might be expected from various types of cutting. It was with this idea in mind that the writer undertook a study of light conditions and forest growth in a virgin Norway pine forest.

The present study does not attempt to take into consideration all factors which affect plant growth, but rather to take measurements of a single factor and to see how these measurements may be correlated with the vegetation present. As shown by Adams (1),<sup>3</sup> air temperature, soil temperature, soil moisture, relative humidity, and evaporation are all changed by thinning a forest stand. All of these factors, as well as light intensity, are more or less directly correlated with solar radiation. If, therefore, within any climatic and edaphic unit area a single factor is to be chosen for correlation with forest growth, light would seem to be the most promising.

Forest trees, however, as shown by Toumey (16) and others, tend to deplete soil moisture more rapidly than less massive forms of vegetation. It should be borne in mind, therefore, that root competition for both moisture and nutrients is an important concomitant factor to be taken into account in determining the significance of the light data obtained in this study. In this connection, however, it may be mentioned that preliminary results in the Lake States indicate that an overstory tends to protect the undergrowth from severe drought injury.

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<sup>1</sup> Received for publication July 25, 1931, issued April, 1932. "Light," in this paper, is used synonymously with solar radiation unless specifically qualified.

<sup>2</sup> The writer wishes to acknowledge his indebtedness to Mary C. Shirley, who assisted him with the vegetation enumeration in the field and with the editing of the manuscript.

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Common Name	Scientific Name
Aster.....	<i>Aster</i> sp. incert
Late aster.....	<i>Aster tardiflorus</i> L.
Harebell.....	<i>Campanula rotundifolia</i> L.
Common pipsissewa.....	<i>Chimaphila umbellata</i> (L.) Nutt
Bastard toadflax.....	<i>Comandra pallida</i> A. DC.
Erect bindweed.....	<i>Convolvulus spithameus</i> L.
Trailing-arbutus.....	<i>Epigaea repens</i> L.
Fireweed.....	<i>Chamaenerion angustifolium</i> (L.) Scop., syn. <i>Epilobium angustifolium</i> L.
Virginia strawberry.....	<i>Fragaria virginiana</i> Duchesne
Northern bedstraw.....	<i>Galium boreale</i> L.
Cream pea vine.....	<i>Lathyrus ochroleucus</i> Hook.
Puccoon.....	<i>Lithospermum canescens</i> (Michx.) Lehm.
Narrowleaf cowwheat.....	<i>Melampyrum lineare</i> Lam.
Fringed polygala.....	<i>Polygala paucifolia</i> Willd.
Rattlesnake-root.....	<i>Prenanthes alba</i> L.
Sidebells pyrola.....	<i>Pyrola secunda</i> L.
Roundleaf pyrola or shinleaf.....	<i>Pyrola americana</i> Sweet
Dwarf dewberry.....	<i>Rubus triflorus</i> Richards
Goldenrod.....	<i>Solidago</i> sp.
American germander.....	<i>Teucrium canadense</i> L.
Early meadowrue.....	<i>Thalictrum dioicum</i> L.
American starflower.....	<i>Trientalis americana</i> (Pers.) Pursh
Cow vetch.....	<i>Vicia cracca</i> L.
Violet.....	<i>Viola conspersa</i> Reichenb.

#### RELATED INVESTIGATIONS

Studies on the growth of plants exposed to different intensities of light indicate that growth is almost directly proportional to the amount of light available up to values of about 700 foot-candles of artificial light or about 50 per cent of full sunlight in latitudes of the northern United States. Intensities higher than 50 per cent sometimes cause a decrease in growth, especially in plants whose natural habitat is in the shade (12). Shantz (11), working in Louisiana, found that several plants produced maximum growth with 15 per cent of full sunlight, while others required 50 per cent or more. Zillich (17), in Germany, found that several weeds produced best growth in 33 per cent light; cultivated plants, on the other hand, produced maximum dry weight in 100 per cent light and showed a marked decrease when shaded. Many others have studied the influence of shading on plant growth and similarly reached the conclusion that slight shading is sometimes beneficial, whereas heavy shading invariably causes a decrease in growth. The amount of shading required to obtain the best growth varies with the latitude and climatic conditions of the station in question.

The effect of shade in natural forest stands has also been studied, but the results are somewhat less consistent. Moore (8) established a series of seed spots in dense shade and in small openings in four forest types of Mount Desert Island, Me. He sowed Norway and northern white pines and white and red spruce seed in prepared spots. Survival was poor for all species in the shade, except in the northern hardwood type. Growth and vigor of the seedlings were in every case markedly better in the small openings.

Pearson (9, 10) studied the growth and survival of western yellow pine in the southwestern United States, with particular reference to the influence of light. He found that after a good seed year, seedlings would come up even more abundantly on the bare areas under the

The common and scientific names of trees, shrubs, and herbs mentioned in this article are as follows:<sup>4</sup>

## TREES

Common Name	Scientific Name
Red maple.....	<i>Acer rubrum</i> L
Paper birch.....	<i>Betula papyrifera</i> Marsh
Jack pine.....	<i>Pinus banksiana</i> Lamb
Norway pine.....	<i>Pinus resinosa</i> Soland
Northern white pine.....	<i>Pinus strobus</i> L
Aspen.....	<i>Populus tremuloides</i> Michx.
Pin cherry.....	<i>Prunus pennsylvanica</i> L f.
Red Oak.....	<i>Quercus borealis</i> Michx f
Bur oak.....	<i>Quercus macrocarpa</i> Michx

## SHRUBS

Alder.....	<i>Alnus rugosa</i> (Du Roi) Spreng and A. <i>incana</i> (L) Moench
Thicket shadblow or June berry.....	<i>Amelanchier oblongifolia</i> (Torr and Gray) Roem
Bearberry.....	<i>Arctostaphylos uva-ursi</i> (L) Spreng
Inland Jersey-tea.....	<i>Ceanothus ovatus</i> Desf
Bunchberry.....	<i>Cornus canadensis</i> L
American hazelnut.....	<i>Corylus americana</i> Walt.
Beaked hazelnut.....	<i>Corylus rostrata</i> Ait
Dwarf bush-honeysuckle.....	<i>Diervilla lonicera</i> Mill
Wintergreen.....	<i>Gaultheria procumbens</i> L
American twinflower.....	<i>Linnaea borealis</i> L, var <i>americana</i> (Forbes) Rehder
Appalachian cherry.....	<i>Prunus susquehanae</i> Willd (syn <i>P</i> <i>cuneata</i> Raf)
Chokecherry.....	<i>Prunus virginiana</i> L
Rose.....	<i>Rosa</i> sp
Red raspberry.....	<i>Rubus idaeus</i> L, var <i>aculeatissimus</i> (C A Mey) Reg and Til
Willow.....	<i>Salix</i> spp
Lowbush blueberry.....	<i>Vaccinium angustifolium</i> Air (syn <i>V.</i> <i>pennsylvanicum</i> Lam)

## HERBS

## PTERIDOPHYTES

Groundcedar.....	<i>Lycopodium complanatum</i> L
Bracken.....	<i>Pteridium aquilinum</i> (L) Kuhn, syn <i>Pteris aquilina</i> L.

## MONOCOTYLEDONS

Sedge.....	<i>Carex</i> sp
Bluebead.....	<i>Clintonia borealis</i> (Ait) Raf
Grass.....	<i>Gramineae</i> sp
Mayflower.....	<i>Unifolium canadense</i> (Desf) Greene, syn <i>Maranthemum canadense</i> Desf.

## DICOTYLEDONS

American wood anemone.....	<i>Anemone quinquefolia</i> L
Pussytoes or everlasting.....	<i>Antennaria</i> sp.
Spreading dogbane.....	<i>Apocynum androsaemifolium</i> L
Wild sarsaparilla.....	<i>Aralia nudicaulis</i> L.
Smooth aster.....	<i>Aster laevis</i> L
Bigleaf aster.....	<i>Aster macrophyllus</i> L.

<sup>4</sup> Nomenclature follows Sudworth (15) for trees and American Joint Committee on Horticultural Nomenclature (8) for other plants

give way to enchanter's nightshade when the light increased some sixfold. Various other plants were associated with the higher light values. There seemed to be a very definite tendency for the vegetation to change with changing light conditions.

Atkins and Stanbury (4) made a study of illumination and plant distribution in spruce, larch, oak, and holm oak woods. Under the shade of a dense spruce stand (*Picea excelsa*) light values were about 1 to 2 per cent, and only ivy and wood sorrel seemed able to survive. Increasing light up to about 9 per cent allowed *Rubus*, ash, hazelnut, and sycamore maple to come in. Larch and oak stands had about 10 to 15 per cent light and supported a good woodland flora. Holm oak reduced the light to 1 to 3 per cent. Under this only ivy seemed able to thrive.

Stallard (14) describes secondary successions in northern Minnesota forests. His paper contains a description of the undervegetation in Norway pine forests and the successional changes brought about by increasing shade. He gives curves showing the annual height growth of Norway, northern white, and jack pines from 1 to 28 years of age, growing in the shade and in the open. The curves for jack pine show rapid falling off in growth with shading, particularly under Norway pine canopies. Norway pine seedlings, on the other hand, appear able to grow at a reasonable rate when shaded by jack pine but not so rapidly as in the open. Northern white pine on heavy soils showed almost as rapid height growth in the shade of hazel, aspen, and birch (10 to 30 per cent light) as in the open. Stallard considers that Norway and white pines form the mature stage in the succession in coniferous forests of Minnesota.

#### THE STAND

This study was carried out on the Chippewa National Forest located in the north-central part of Minnesota. Along the shores of Cass Lake and Pike Bay are 10 sections of Norway pine forests which have been undisturbed by logging operations, except to remove dead and dying trees at 5-year intervals. All this area has been subject to fires at various times during the past. However, most of the fires have been comparatively light, since none of them killed many of the large trees. The trees average more than 200 years in age and are fine, tall specimens, typical of the virgin Norway pine stands of the Lake States (Fig. 1.) The trees average about 90 feet in height and in many places stand as dense as their crowns will permit. Numerous openings up to 300 or more feet in diameter can be found in the forest. They were perhaps caused by fire, wind, or other destructive agencies. The undergrowth is composed mostly of woody shrubs, among which bearberry and blueberry are dominant. Many of the openings are growing up to vigorous stands of young Norway pine and others to hazelnut, oak, and paper birch. The lower vegetation throughout is fairly luxuriant and is indicative of the presence of ample moisture and light for satisfactory plant growth. Scattered through the Norway pines are occasional white pines, usually larger and overtopping the Norways. Under the old white pines there can almost invariably be found numerous white pine seedlings. Where Norway and white pine seedlings have an equal chance for establishment white pine seems to be more abundant. Jack pine seed trees

clumps of trees than in the openings. Practically all of those in dense shade died, however, or became unthrifty within four or five years. Even on the north side of tree groups survival was poor and annual height growth was only 0.4 inch to 3 inches, as against 3 to 6 inches for seedlings of the same age in adjacent openings.

Gast (5), during an entire growing season, measured the total radiation received at three different stations where cuttings had recently been made. These measurements were correlated with the growth of the leaders of white pine saplings. He found the leader growth to be apparently directly proportional to the radiation, up to the intensity of full sunlight. The average leader growth of white pine trees 11 years old under a canopy transmitting 27 per cent light was only 2 inches per year and showed little increase with increasing age of the trees. He believes this to represent about the minimum radiation intensity for growth of white pine. Since Gast's measurements of sunlight were made by a continuously recording mechanism operating throughout the summer, his percentage values are higher than those of most workers who make readings only on bright days.

Holch (7) grew bur oak, red oak, hickory, linden, and walnut on three forest sites—the prairie, a bur oak forest, and a linden forest. The light values averaged 10.4 per cent for the oak and 3.5 per cent for the linden station on the basis of the prairie station as 100 per cent. The growth of both roots and shoots was greatest for all species in the prairie and least in the linden forest, where most of the plants died before the end of the third season. Photosynthesis was very rapid in all species at the prairie station, was weak at the bur oak station, and very weak at the linden station. Evaporation, transpiration, and soil and air temperatures decreased with decreasing light, and soil moisture increased. Soil moisture was, however, sufficient in all three sites. Under the conditions of Holch's experiment, survival, growth, and photosynthesis were directly correlated with light and inversely correlated with available water content of the soil.

Grasovsky (6) placed northern white pine, Norway pine, hemlock, red oak, and chestnut oak seedlings in boxes with a window in one end. At the end of 10 months those so far removed from the window that they received no more than 300 foot-candles of illumination during the entire period were still alive and apparently in vigorous condition. No measurements of dry weight were made to determine whether the plants had actually grown. Grasovsky states that only a moderate increase over the minimum light-intensity requirement for survival was necessary to maintain growth and that thereafter the effect of added light on growth was not at all proportional to the intensity. Measurements of the light intensity under white pine canopies showed it to be in all cases in excess of the minimum light requirements of the plants tested. He concludes that the intensity of light reaching the forest floor is not the limiting factor in accounting for the presence or absence of forest reproduction in the fully stocked stands where the investigation was conducted. His conclusions are somewhat at variance with those cited above. The investigations, however, were carried out under conditions sufficiently different to account for any discrepancies in results.

Atkins and Poole (3) studied the correlation between light intensities and plant distribution in an old garden. In the deepest shade they found only straggling branches of English ivy which tended to

## METHODS

All field work was carried out doing the summer of 1930. Fifty plots, approximately one-tenth acre in size, were laid out in groups of three to five and chosen to show differences in forest reproduction. A typical group would contain one plot without forest reproduction, one plot in which the forest reproduction was present but was making very poor growth, one plot in which the forest reproduction was making rapid growth, and perhaps one plot occupied by aggressive brush species. A temporary stake marked the center of each plot. The soil was examined to a depth of 8 inches. General notes were taken on the character of the stand, the date of the last fire, and the general character of the undergrowth.

Two quadrats, 0.001 acre each, were laid out at equal distances on opposite sides of the center stake. On these quadrats all coniferous reproduction was counted and the average age and height determined. The mean annual height growth of the conifers was found by dividing the average height by the average age.

All species of plants growing on each plot were listed and their relative abundance estimated in three classes. Class 1, less than one-twentieth of the area occupied; class 2, one-twentieth to one-fifth of area occupied, and class 3, more than one-fifth of area occupied.

## LIGHT MEASUREMENTS

Measurements of total and diffuse radiation on cloudless days were made with a thermopile (13) at 10 points uniformly distributed over the area of each plot. The measurements were converted to percentages of the radiation intensities in the open and averaged for the entire plot. It was originally intended to use the readings of diffuse light as a basis for correlation, but it was found that a very considerable amount of direct sunlight is reflected from leaves, hence measurements of diffuse light included not only the sky light which penetrated the canopy but in addition a large amount of reflected sunlight. It was also found that measurements of diffuse light were only roughly correlated with measurements of total sunlight. Over a range of from 30 to 100 per cent of total sunlight, diffuse light changed from only 52 to 66 per cent.

It was felt, therefore, that the measurements of diffuse radiation made did not give an accurate evaluation of the light available to the undervegetation.

Measurements of total radiation, on the other hand, show such great variability that it is difficult to get a reliable estimate of the light available on a given area. The standard deviations for 10 readings ranged from 1 to 14 per cent.

TABLE 1—Standard deviations of light measurements by 10 per cent intensity classes

Intensity class	Mean value	Plots	Standard deviation of mean	Intensity class	Mean value	Plots	Standard deviation of mean
	<i>Per cent</i>	<i>Number</i>			<i>Per cent</i>	<i>Number</i>	
0-9 per cent.....	4.0	5	1.1	50-59 per cent.....	56.0	8	4.3
10-19 per cent.....	17.0	1	4.0	60-69 per cent.....	63.5	6	4.9
20-29 per cent.....	25.0	9	2.8	70-79 per cent.....	75.0	2	8.7
30-39 per cent.....	36.0	5	4.6	80-89 per cent.....	89.7	1	9.0
40-49 per cent.....	44.0	5	5.1	90-100 per cent.....	92.0	2	1.0

are less common than white pine and are usually found in groups in the larger openings or in more or less pure stands along the periphery of the old Norway pine. Where jack pine seed is available the seedlings are usually present and grow quite rapidly.

Wherever pine reproduction has become sufficiently well established to exclude most of the other vegetation it is entirely too dense for good growth. On practically all the plots examined the young pines were too crowded to make rapid growth or they were suffering from competition with other plants. Apparently not until the young saplings have attained a height of 10 to 20 feet is there sufficient expression of dominance for the leaders to make rapid growth. On only two of the plots included in this study had the stand reached this stage, and on each of them the dominant trees showed recent



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FIGURE 1—Typical virgin Norway pine stand, with young Norway reproduction in the openings. These saplings are about 18 years old and 4 to 5 feet high.

annual height increments of 1 foot and more. The average height growth for trees less than 6 feet high in crowded stands is about 0.3 foot.

The soil is of fluvioglacial origin. It is classed as fine sand and is weakly podsolized. The humus layer is usually less than 2 inches thick and tends to decompose rather rapidly. The gray leached layer is usually 6 to 8 inches thick, and the brown layer generally extends to a depth of 2 or more feet. During the dry period in the summer of 1930 practically all the available moisture was withdrawn to a depth of 2 feet. Below this depth the soil was in most places quite moist. Most of the plots examined lay less than 20 feet above the lake level.

A good crop of cones was produced by the old trees in the fall of 1930, and there is every reason to suppose that ample seed has been produced at intervals of from three to seven years during the past century. Absence of forest reproduction on any particular spot can, therefore, scarcely be attributed to lack of available seed in the past.

arbitrarily represented by averaging its class numerals 1, 2, and 3 for all plots in their light class. Plots on which a given plant did not occur were not used in determining the abundance for the class. Frequencies were determined by dividing the number of plots on which a given plant occurred in an intensity class by the number of plots in the class. Abundance and frequency curves are shown in Figures 5, 6, and 7, with numerals showing the number of plots used

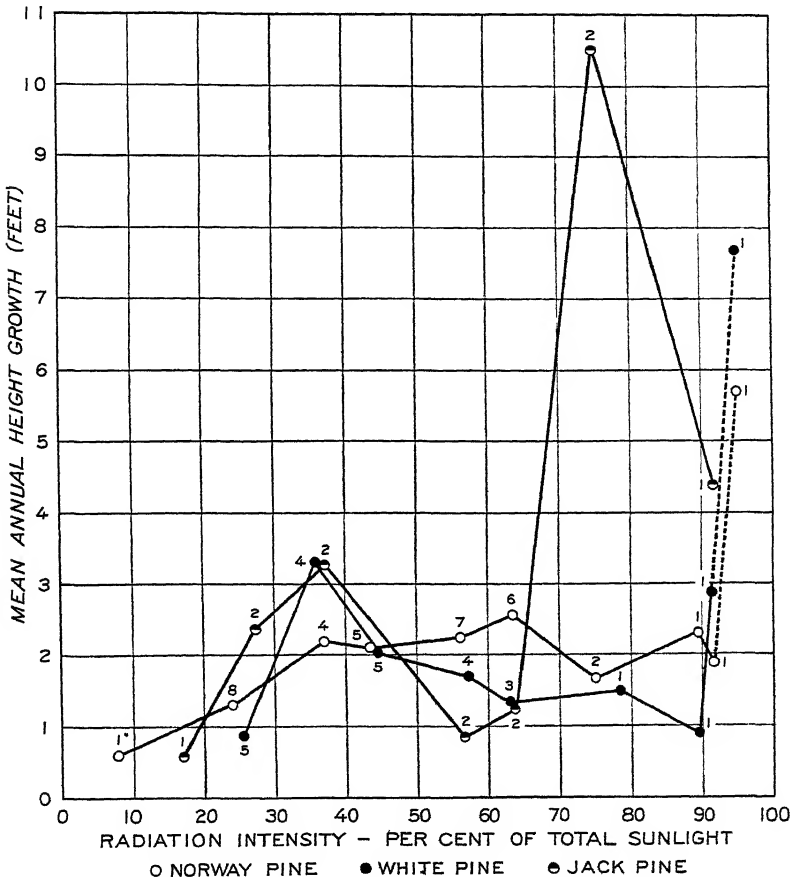


FIGURE 3—Correlation between light intensity and the mean annual height growth of young Norway, white, and jack pines growing in a virgin Norway stand. Numerals indicate number of plots in each class.

in determining a point. The weight on the abundance point divided by the weight on the corresponding frequency point gives the frequency. Only plants occurring on at least 10 plots are used in these figures.

#### LIGHT AND NATURAL REPRODUCTION

The number of trees per mil-acre is plotted against light in Figure 2. The poor showing of jack pine may be the result in part of insufficient light, but undoubtedly the chief reason for its sparsity is lack of seed trees. The comparatively small number of white pines established

The plots were grouped in 10 per cent light-intensity classes, depending upon the mean light values for each plot. Standard deviations were again calculated for each group of plots. These are given in Table 1. The points shown on the curves in Figures 2, 3, and 4 may therefore be considered to be in error as much as 5 per cent either way in light measurements. This is probably considerably less than the error in sampling the vegetation and coniferous reproduction.

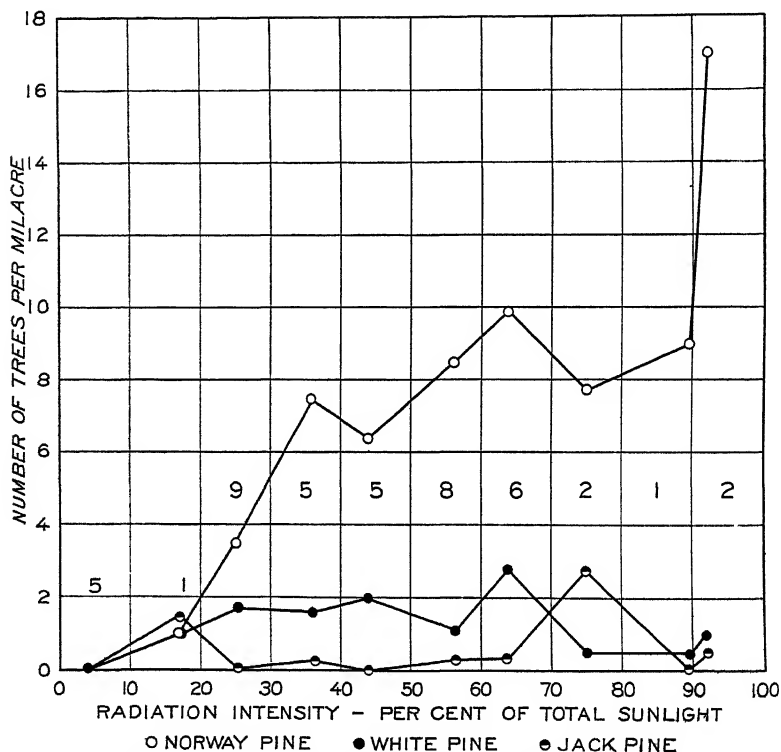


FIGURE 2—Correlation between light intensity and abundance of Norway, white, and jack pines

#### ANALYSIS OF MATERIAL

Height measurements and number of trees per mil-acre (one-thousandth acre) were averaged for each plot. These were then plotted against light intensity and averaged by 10 per cent classes (Figs. 2, 3, and 4). Plots showing evidence of fire since 1918 were not included in determinations the results of which are shown in these figures. The number of trees per mil-acre in any class is obtained by averaging the number per mil-acre found on each plot in the class. The number of plots occurring in each class is shown by a numeral on the graph. The mean annual height growth is likewise averaged by light-intensity classes. In this case, however, plots having no coniferous reproduction were not used in finding the average point. The weight on a point shows the number of plots used in determining its position. The abundance of a plant in any 10 per cent light class is

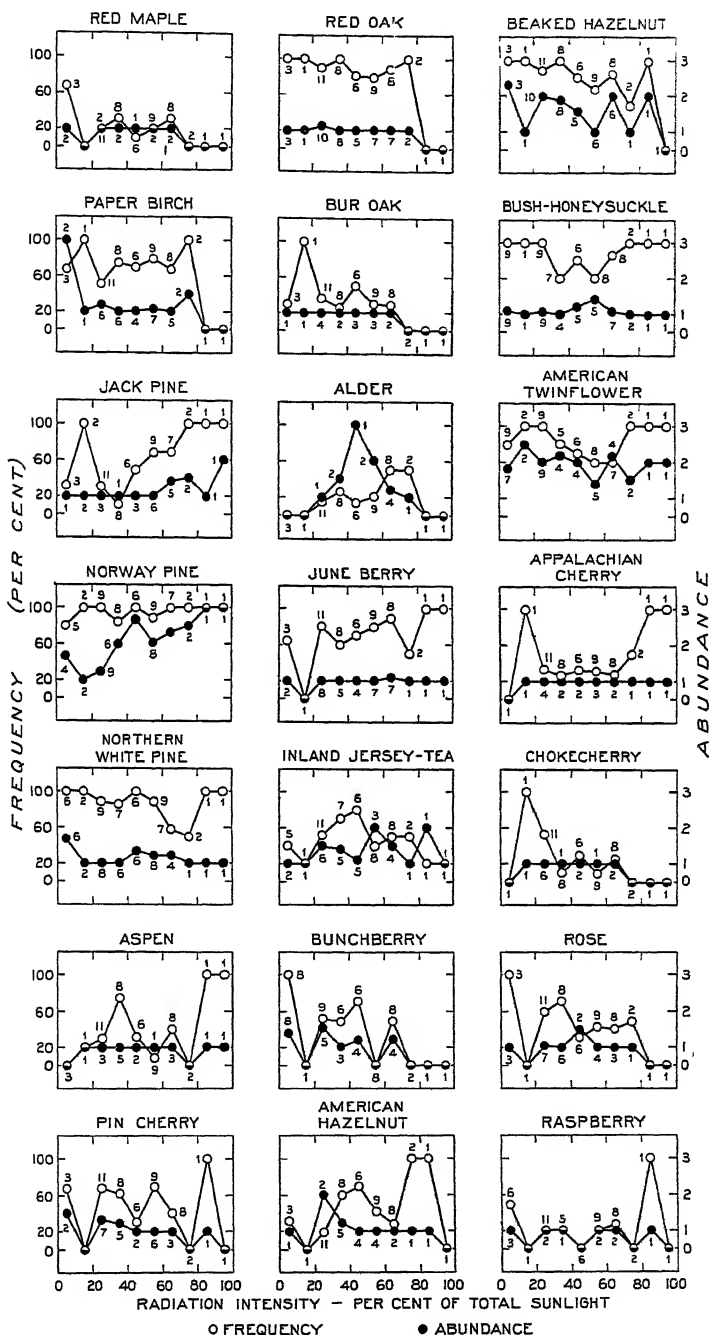


FIGURE 5—Correlation between light intensity and the frequency and abundance of various woody plants found growing in a virgin Norway pine stand. Numerals indicate number of plots used to determine the point.

is also due to lack of seed. The curve for Norway pine, on the other hand, well illustrates the correlation between light and establishment. None of the three species mentioned was able to become established where less than 5 per cent light was available. At 17 per cent light, establishment was fair—the equivalent of 3,000 trees per acre, which is about the minimum number for satisfactory natural reproduction. Any light value higher than 35 per cent seemed to be excellent for the establishment of Norway pine seedlings, as all plots averaged more than 6,000 trees per acre.

If Norway pine seedlings are not present on areas receiving 35 per cent light or more, no method of cutting the old stand is likely to better matters, since factors other than light are operative in keeping the pine out. The lowest average light intensity on any plot shaded only by old Norway pine was 17 per cent. This plot had the equivalent of

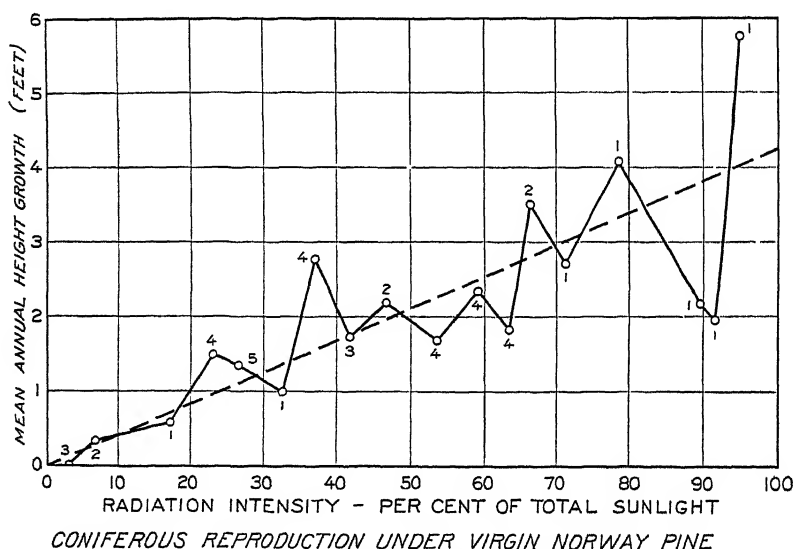


FIGURE 4—Correlation between light intensity and mean annual height growth of stands of young Norway, white, and jack pines. Numerals indicate number of plots in each class.

3,500 pine seedlings per acre. It seems, therefore, that virgin Norway pine stands are not likely to be so dense as completely to exclude coniferous reproduction.

Although the shade cast by the old Norway pines themselves may never be too dense for the establishment of reproduction, that cast by an understory of hazelnut, birch, alder, and other shrubs may quite effectively exclude pine seedlings. Fifty-five measurements taken of light beneath brush canopies gave values ranging from 0.7 to 15.8 per cent, with a mean of 4.4 per cent and a median of 3.5 per cent. Only rarely are pine seedlings able to grow through an understory of such density. In practically every case where the brush formed a continuous canopy, pine reproduction was either absent or in very poor condition. The whole study points to the importance of the undergrowth in determining coniferous establishment in virgin stands.

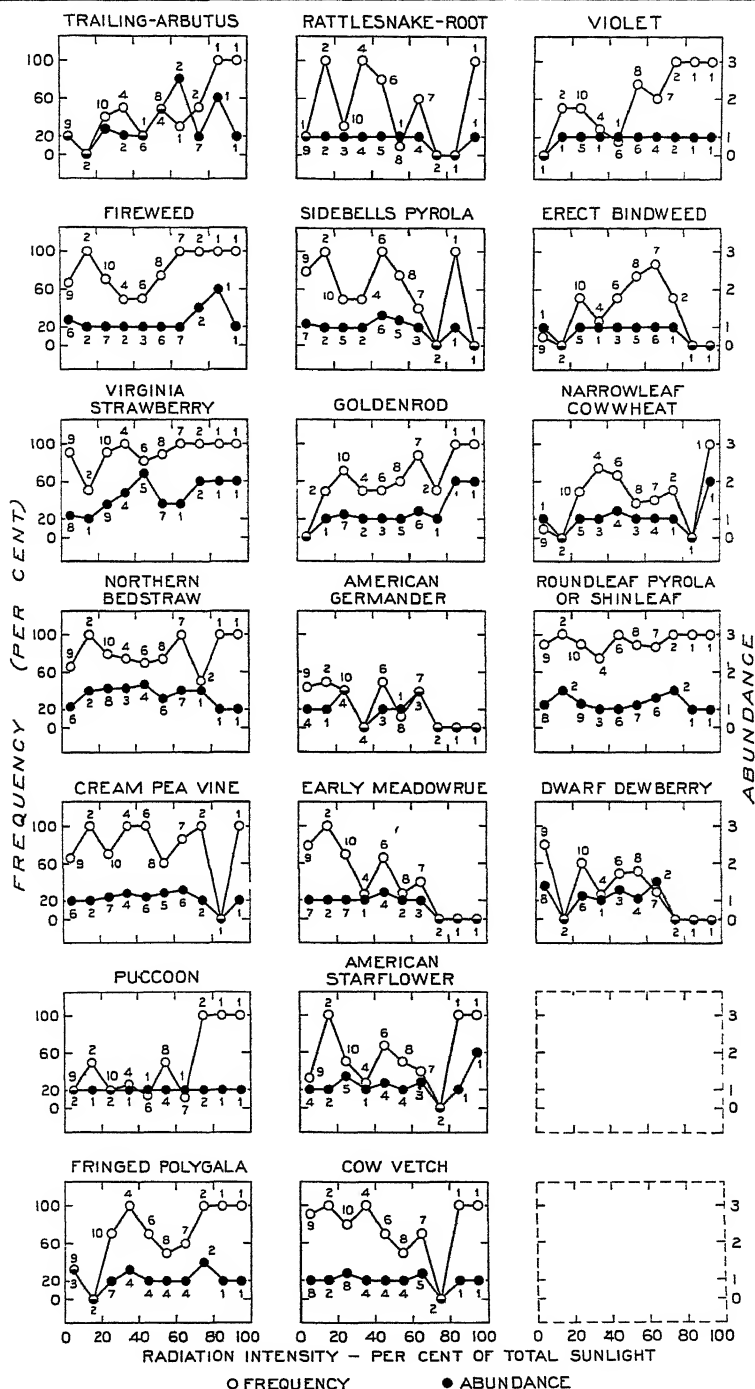


FIGURE 7—Correlation between light intensity and the frequency and abundance of various herbaceous plants growing in a virgin Norway pine stand. Numerals indicate number of plots used to determine the point

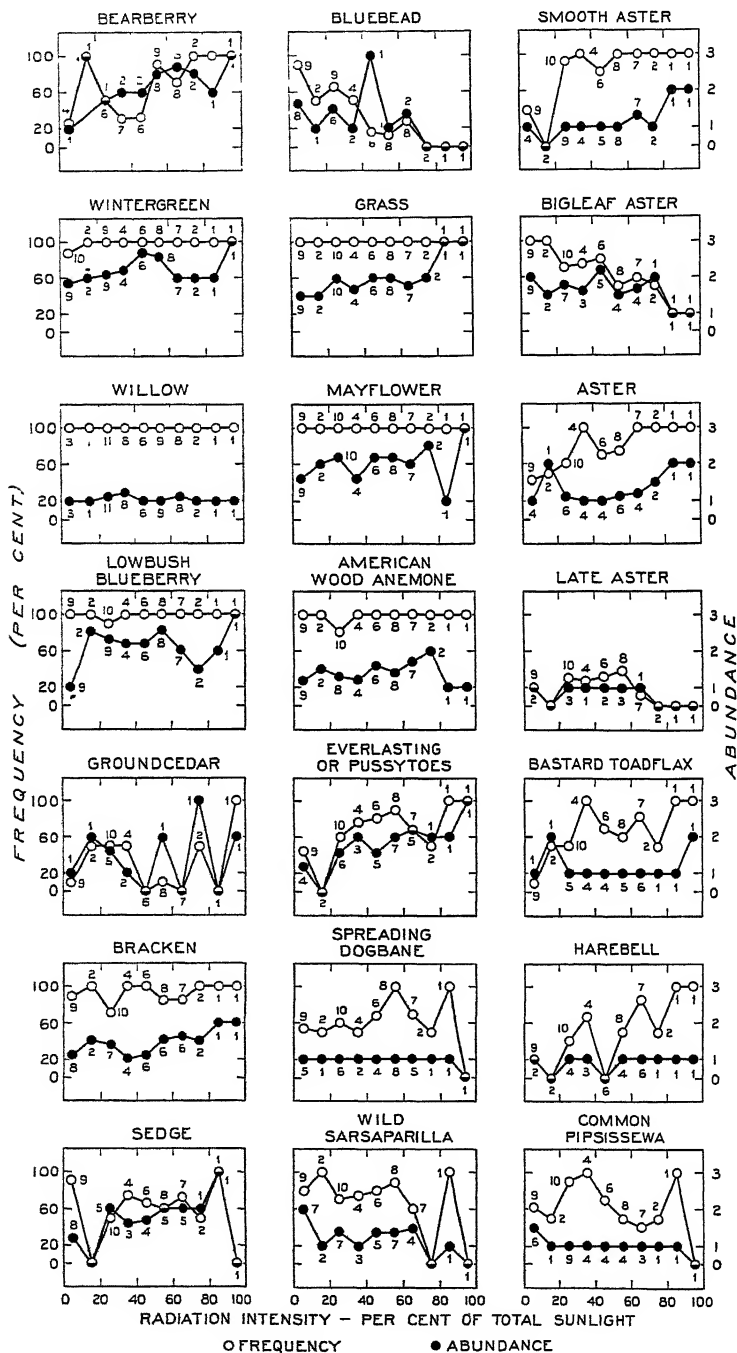


FIGURE 6—Correlation between light intensity and the frequency and abundance of various woody and herbaceous plants growing in a virgin Norway pine stand. Numerals indicate number of plots used to determine the point.

slow to insure thrifty development of the young stand. In fact, trees showing only one-half inch yearly growth are generally extremely unhealthy or actually dying

### LIGHT AND THE GROUND COVER

The frequency and abundance of other plants are plotted against light intensity in Figures 5, 6, and 7. The curves given are based on estimates rather than accurate counts; hence only general trends are considered significant. In studying these curves, particularly the frequency, attention must be paid to the weights of the points, as any point with a weight of 1 can have only frequencies of 0 or 100 per cent and one with a weight of 2 may have in addition only 50 per cent.

TABLE 2.—*Classification of ground cover as to abundance under different light intensities*

Cover never abundant	Cover abundant at high intensities	Cover abundant at intermediate intensities	Cover abundant at low intensities
Paper birch. Red maple Bur oak Red oak Aspen Thicket shadblow or June berry Pin cherry Appalachian cherry Chokecherry Willow Red raspberry Spreading dogbane Late aster Harebell Common pipsissewa Cream pea vine Puccoon Fringed polygala Rattlesnake-root Roundleaf pyrola or shinleaf Sidebells pyrola Early meadowrue Cow vetch Violet Erect bindweed Bastard toadflax. American starflower Narrowleaf cowwheat	Jack pine Norway pine Inland Jersey-tea Bearberry Bracken Sedge Grass Mayflower American wood anemone Pussytoes or everlasting Aster Smooth aster Virginia strawberry. Goldenrod Fireweed.	White pine Alder American hazelnut Beaked hazelnut Dwarf bush-honeysuckle Rose Wintergreen Lowbush blueberry Wild sarsaparilla Bigleaf aster Trailing-arbutus Northern bedstraw Dwarf dewberry.	Bunchberry American twinflower Groundcedar. Bluebead American germander

TABLE 3.—*Classification of ground cover by tendency to high frequency of occurrence under different light*

Indefinite	Greatest at high intensities	Greatest at intermediate intensities	Greatest at low intensities
Pin cherry Red oak Dwarf bush-honeysuckle Wintergreen Appalachian cherry Red raspberry Willow Lowbush blueberry Grass Mayflower Bracken American wood anemone Cream pea vine Puccoon Roundleaf pyrola or shinleaf	Paper birch Jack pine Norway pine Alder Thicket shadblow or June berry Bearberry. American hazelnut Sedge Pussytoes or everlasting. Smooth aster. Aster Harebell Trailing-arbutus. Fireweed Virginia strawberry Goldenrod Violet	Red maple Aspen Inland Jersey-tea Rose Spreading dogbane. Late aster Common pipsissewa Bastard toadflax Erect bindweed Narrowleaf cowwheat Fringed polygala Rattlesnake-root Sidebells pyrola American starflower	White pine Bur oak Bunchberry Beaked hazelnut American twinflower Chokecherry Groundcedar Bluebead. Wild sarsaparilla Bigleaf aster Northern bedstraw American germander Early meadowrue Cow vetch Dwarf dewberry.

The minimum light value in which Norway and white pines can exist seems to be between 1 and 5 per cent. Twenty-one measurements of light beneath dense Norway pine reproduction gave values ranging from 0.9 to 8.2 per cent with a mean of 3.4 per cent and a median of 3.0 per cent. No young seedlings were actually growing where these low values prevailed, yet the saplings themselves were able to reduce the light to that value. If it is assumed that green pine needles can not persist long in light values too low to supply energy for their own photosynthetic needs, then the minimum light requirements for survival would be approximately equal to the intensity prevailing beneath the very dense stands. This value is, however, probably nearer the minimum requirement for survival where other factors are favorable, and would be, therefore, much lower than the value required for establishment and growth of pines in the forest.

#### LIGHT AND HEIGHT GROWTH

In Figure 3 the mean annual height growth for Norway, white, and jack pines is plotted with light intensity as abscissas. Both white pine and jack pine were usually sparse in the understory, hence the curves show great irregularities. Norway pine, on the other hand, shows a fairly regular trend. It can be seen that up to about 63 per cent light, at which point Norway pine showed a maximum, the growth increases fairly regularly with increasing light. Jack pine shows a maximum at 75 per cent and white pine at 36 per cent. The ability of a species to attain maximum height growth at low light values may be considered as a measure of its relative tolerance. The relative tolerance of the three species, as determined by this method, is, in decreasing order, white pine, Norway pine, and jack pine. This is the order of tolerance commonly accepted in this region.

At this point it is well to consider the actual maximum values. White pine showed a maximum mean annual growth of 0.33 foot or 4 inches, Norway pine of 0.25 foot or 3 inches; and jack pine of 1.05 feet or 12½ inches. Except for jack pine, the maxima are low. They do not represent the maximum possibilities of the species, but rather the average maximum growth attained in crowded stands beneath an upper canopy. One plot, located in the center of an opening about 200 feet in diameter, is covered with a dense stand of Norway and white pines approximately 26 years old and 20 to 30 feet in height. White pine has grown more rapidly than Norway pine and apparently will soon almost completely occupy the area. The true maximal height growth, therefore, for Norway and white pines is much greater than 3 to 4 inches a year and seems to occur in full sunlight rather than in shade. This is indicated by the last point on the curves of Figure 3.

In Figure 4, the mean annual height growth of Norway, white, and jack pines is weighted for the actual number of trees of each species on each mil-acre quadrat. This gives a better picture of the actual growth of the stands of young reproduction, since the species which had gained ascendancy on a given plot seemed to be related to chance as well as to light values. A straight line is drawn on the chart to show how nearly the growth is proportional to the light intensity.

Approximately 35 per cent light appears to be necessary for reasonably good growth. In light values below 20 per cent growth is too

Virgin Norway pine stands 200 years old or older are not likely to be so dense that Norway seedlings can not grow underneath the old trees. If, on the other hand, alder, hazelnut, birch, and other plants become established in the openings in a virgin stand, they may quite effectively exclude all conifers. Removing the remaining old trees from over the underbrush is not likely to result in conifers becoming established unless the brush cover is broken up by logging operations. In marking trees for cutting at the end of a forest rotation, attention should be paid to the undergrowth as well as the mature trees, as a stand of shrubs and aggressive herbs may rapidly take over the area before conifers can be established. On areas devoid of reproduction and having 35 per cent or more light, some other treatment than cutting the old stand should be given to bring in the desired stand of young trees.

The gradual enlargement of small openings in which reproduction is already established would seem to be the ideal method not only for getting a good establishment of reproduction in an old stand but also for providing best conditions for its subsequent survival and growth. Such a method, which is essentially the group selection system, also tends to discourage the active competitors of conifers which seem no more able to thrive beneath a dense canopy of young pine than the pine is able to thrive beneath dense underbrush.

These recommendations as to proper silvicultural treatment are at present offered chiefly as suggestions, since their application to areas outside the stand actually investigated has not been demonstrated.

#### SUMMARY AND CONCLUSIONS

In a virgin Norway pine stand studied in relation to light and to other vegetation, approximately 35 per cent light, or a crown density of about two-thirds, seems to offer satisfactory conditions for the establishment of Norway pine seedlings. Light values below 17 per cent result in uncertain establishment. The number of trees per acre seems to continue to increase with the light up to the intensity of full daylight.

The height growth of Norway pine increased with increasing light up to 63 per cent. White pine showed a maximum growth at about 36 per cent light, and jack pine at 75 per cent. These maxima are believed to be apparent maxima applying only to the conditions in the stand studied. The fact that white pine attains maximum height at lower values than Norway pine, and Norway pine at lower values than jack pine indicates that white pine is the most tolerant of shade and jack pine least. This is in agreement with current opinions based upon other methods of study.

When the growth of the stand is considered instead of the growth of individual species, there is a more definite correlation between the light intensity and height growth, which tends to approach a linear relationship.

The light intensities commonly prevailing in virgin stands of Norway pine are not likely to be too low for the establishment of reproduction. On the other hand, understories of hazelnut and other shrubs, which reduce the light to less than 5 per cent, quite effectively exclude coniferous seedlings.

An examination of the curves in Figures 5, 6, and 7 shows that several species exhibit the same general tendencies in their response to light. In Table 2 they are separated into four groups—those never abundant, those most abundant at high light intensities or tending to become more abundant with increasing light, those most abundant at intermediate light intensities, and those most abundant at low light intensities. This table shows also that the number of species in each group decreases with decreasing light intensities.

The frequency of plants in a given light intensity class is perhaps more subject to chance variation than the abundance, hence, the frequency curves show more irregularities. Some curves are too irregular to show any definite trend. In Table 3 the plants are grouped according to their tendencies to be most frequent at high, intermediate, or low light values.

Examination of Tables 2 and 3 will show that certain species appear to be misplaced. Aspen, beaked hazelnut and bur oak are ordinarily considered intolerant and are usually found growing best on moderately heavy soils. When these plants occur in Norway pine forests they appear to do better in the shade. This condition would probably be reversed on a better soil.

#### COMPETITORS OF CONIFERS

The most serious competitors of young conifers starting under the old Norway pine stand are beaked and American hazelnut, inland Jersey-tea, bracken, grass, sedge, lowbush blueberry, and bearberry. Of these, hazelnut and lowbush blueberry seem to thrive best in light shade, but the others increase in abundance and in vigor with increasing light. As Norway pine does very poorly in less than 35 per cent light, it would seem best to maintain intensities of about 35 to 50 per cent light (which corresponds to a crown density of about one-half to two-thirds in virgin stands) until the Norway pine is sufficiently well started to be out of danger from competition by grass, sedge, bearberry, and blueberry, then to increase the light value by degrees up to 100 per cent. Of course, regulation of crown density alone will not free Norway pine from competing vegetation, but, by maintaining a crown density which is more favorable for Norway pine than for some of its active competitors, the pine should have a better chance to gain ascendancy.

#### RELATION OF LIGHT STUDIES TO SILVICULTURE

The results of this study indicate that both the establishment and growth of young pines in a virgin Norway pine stand are definitely correlated with light intensity. Individual plots show considerable deviation from the general average, but these deviations are due in large part to inaccuracies in sampling the light intensity, inaccuracies in sampling the coniferous reproduction, and certain extraneous factors, chief of which is the competition of other vegetation. The study also indicates that light as a growth factor operates in the same way in the forest as in carefully controlled experiments with artificial conditions. The full effect of added light is, however, not always utilized in the forest because other growth factors may not be favorable.

# GROWTH RECORD OF FERTILIZED APPLE TREES GROWN IN METAL CYLINDERS<sup>1</sup>

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## INTRODUCTION

In 1908 the Pennsylvania Agricultural Experiment Station began a series of fertilizer experiments in commercial apple orchards in several sections of the State. The results (7, 8, 9)<sup>2</sup> were so variable that in many of the orchards it was difficult to find significant differences between contrasted treatments. Studies of these orchards and of the resulting data indicated that four factors were chiefly responsible for the variability of the results: Initial differences in soil fertility; differences in methods of management, especially the use of sod as compared with tillage; slope, which not only created initial fertility differences, but continued to accentuate them during the continuation of the experiment, and genetic differences affecting the vigor of the seedling roots.

## METHODS OF EXPERIMENTATION

In 1918 it was decided to begin at State College a fertilizer experiment in which as many as possible of these variables would be eliminated or considerably reduced in effect through the use of metal cylinders to confine the roots in uniform soil. At the time this experiment was being planned, the Delaware Agricultural Experiment Station was growing peach trees in large concrete pits and the Florida station (3) had citrus trees growing in eight galvanized-iron tanks, 5 feet 3 inches by 4 feet; but, as far as the authors know, no successful attempt had been made to grow apple trees to maturity in this manner. While this experiment was under way, a number of experimenters successfully used dwarf apple trees in pots in fertilizer studies. The recent work of Davis is an example (4).

To secure greater uniformity of root growth it seemed desirable to use vegetatively propagated roots of a single clone. Through the cooperation of R. G. Hatton, of the East Malling Research Station, Kent, England, the requisite number of Malling Type 12 apple roots were forwarded in January, 1920. Unfortunately, this shipment was lost in transit. The following year Professor Hatton sent a second shipment of the same stock, but was obliged to include stock of two ages—some that had been removed from the mound layer and grown one year in the nursery row and some that had just been separated from the mounds.

These roots were planted in a nursery at State College in the spring of 1921. Ten days later the older stocks were cut off and whip grafted with scions from a 10-year-old Stayman Winesap tree in the

<sup>1</sup> Received for publication July 28, 1931, issued April, 1932. Technical Paper No. 537 of the Pennsylvania Agricultural Experiment Station.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 265.

In virgin Norway pine forests, the presence of an understory of shrubs is just as important in determining the establishment of pine seedlings as the density of the old stand

The group selection cutting system most nearly duplicates the conditions in a virgin stand which appear most satisfactory for the establishment and growth of the young trees

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Because of the limited number of stocks available, it was not possible to make a close selection of the planting material. However, the smaller number needed when the two outer rows were planted made it possible to select fairly uniform material for these rows. The coefficient of variability with respect to weight of trees for the 28 in the middle four rows was  $31.9 \pm 3.2$ , for the outer two rows,  $8.5 \pm 1.1$ .

The fertilizer treatments were planned to run north and south, with six trees in the row and seven rows available for contrasting treatments (Fig 1). Table 1 shows the attempt that was made to smooth off the variability of the material at the first planting. The two lightest trees were planted in the two central rims of the east row and the two heaviest in the two adjoining rims of the same row. There is a progressive increase from east to west in the tree weights in the central two rows of trees, and from west to east in the adjoining two rows. Thus there is a high degree of variability among the trees in the east row but four quite uniform trees in the west row.

The entire area received the same treatment until May, 1924, when the trees had become well established. Each year the trees were hoed during the spring and summer, and winter cover crops were grown.

Early in the planning of this experiment it was seen that many of the problems could be solved only by chemical studies. William Frear, chemist of the Pennsylvania Experiment Station, made a preliminary study of available methods of attack, with the assistance of Walter Thomas, of the Department of Agricultural and Biological Chemistry. Since the death of the former in 1922, Doctor Thomas has had charge of the chemical research connected with this project, while the Department of Horticulture has had charge of the care of the trees and the taking of all field records.

The only pruning given the trees while they were under uniform treatment was to remove the wood needed for chemical analyses, most of the material being taken from the trees that had made the heaviest growth. The correlation between the total branch elongation of the trees for 1922, 1923, and 1924 and the length of the prunings removed from the trees was  $0.37 \pm 0.09$ . This heavier pruning of the more vigorous trees helped to increase the uniformity of the block.

As long as this experiment was in progress, trunk circumference and branch elongation were measured each year, but not spur growth. All growths under 5 cm were considered potential spurs. In 1922 the leaves were secured at leaf fall and weighed. In after years leaf samples were weighed, but no estimates of the total leaf area or weight were made until 1927 at the time the trees were dug up.

The coefficient of variability of the first year's total branch elongation for the 42 trees was  $46.3 \pm 4.1$ . This included the 1922 growth of rows B through E and the 1923 growth of rows A and F. When these 1923 records of rows A and F were used with the same year's records for the other rows, the coefficient of variability for total branch elongation was  $24.2 \pm 1.9$ , and for trunk diameter,  $12.9 \pm 0.97$ . These figures indicate that the outer rows, though planted a year later, are fairly comparable to the inner four rows because of their larger size at planting.

A mixture of bluegrass and timothy was seeded in half of the rims on May 27, 1924. The coefficient of variability of the branch elonga-

college orchard, which, in turn, had been propagated from a tree used in one of the fertilizer experiments begun in 1908. In the summer of 1921 the remaining stocks were budded from the same tree

In the meantime, 42 cylinders, or "rims," of  $\frac{3}{8}$ -inch boiler plate 5 feet across and  $5\frac{1}{2}$  feet deep had been secured. These were sunk in the ground to within 6 inches of the top at the corners of 20-foot squares, making 6 rows of 7 cylinders each. The area of each rim was approximately one twenty-two-hundredths of an acre. Each rim held about 5 tons of soil. The bottoms of the cylinders were not closed, but were set on several inches of coarse crushed limestone. The first 3 or 4 inches of the filling were of this material. It was expected that this layer of stone would prevent the downward movement of the tree roots, but this proved not to be the case.

The soil used to fill these cylinders was taken from an area on the college farm that, as far as could be determined, had received little or no fertilizer. The soil was dug in three layers, the first, 9 $\frac{1}{2}$  inches deep; the second, 9 $\frac{1}{2}$  to 18 $\frac{1}{2}$  inches; and the third, from 18 $\frac{1}{2}$  to 60 inches. Each layer was thoroughly mixed and placed in the rims in its original order. Water was run in when the soil was placed in the rims to assist in compacting it. This work extended through the fall of 1919 and the spring of 1920.

The soil used is classified as a Hagerstown silty clay loam. It has been formed in place by the weathering of the limestone of the lower Silurian formations. As the rims were filled, samples were taken for chemical and mineralogical analyses. The results of these examinations have been presented by Thomas (10). The soil is rich in a wide range of the rarer elements. Phosphorus is low, the analysis showing only about 0.1 per cent  $P_2O_5$ , while potassium is high, with  $K_2O$  averaging over 4 per cent.

Because of the loss of the first shipment of stocks, trees were not available for planting in the rims until the spring of 1922. In the meantime, crops were grown in the rims and worked into the soil to improve its fertility.

#### PLANTING AND EARLY CARE OF TREES

The first planting was made in May, 1922. The four inner rows of seven rims each were filled with the older stocks which had been whip grafted the previous spring. On April 19, 1923, the two outer rows were planted with the stocks that had been budded. Table 1 shows the weights of these trees at planting and their location.

TABLE 1.—Weights of trees at time of planting

Row	Weight (grams) of trees in row indicated					
	F <sup>a</sup>	E	D	C	B	A <sup>a</sup>
No 1.....	375	130	130	120	135	385
No 2.....	370	140	115	115	135	350
No 3.....	350	150	110	110	175	345
No 4.....	340	180	100	100	180	335
No 5.....	325	180	90	<sup>b</sup> 430	195	315
No 6.....	315	210	85	85	195	310
No 7.....	290	215	75	75	220	902

<sup>a</sup> Rows A and F were planted in 1923, 1 year later than the other rows. The later-planted trees were larger at the time of planting.

<sup>b</sup> This tree was also planted in 1923. It replaced the original tree which had been badly injured with a cultivating tool.

Nitrogen and phosphorus were employed as single element treatments but potassium was not. The Hagerstown soil used in this experiment is well supplied with potassium, and none of the orchard tests previously conducted in this State had shown any clear evidence of a potash response.

Numerous field experiments have indicated that the nature of the fertilizer response is very materially altered by the presence or absence of grass in the orchard. Because of this fact, the south half of each row of rims (trees D, E, and F) was seeded to bluegrass on May 27, 1924.

The first fertilizer treatment was not made until the spring of 1925; hence there was nearly a year during which half of the block was under an unfertilized sod. It was not expected that this new sod would materially influence the growth of the trees in such a relatively short time, but Table 4 shows that it did to a significant extent in the majority of the rows.

The low odds in row 5 were due to the fact that one of the cultivated trees had been replanted. In row 7 one of the largest trees growing in sod had been killed back to within a few inches of the bud during the winter of 1923-24, and consequently made a heavy renewal growth in 1924. Table 4 shows that the trees growing in sod started the period of fertilization with a slight handicap.

The first application of fertilizer was only two-thirds of the amount intended to be used as an annual application. In spite of this reduction, the concentration of these amounts in the small area of a single rim resulted in serious burning of the grass. For this reason the other third was not put on in 1925, and in later years the amounts used in a single application were further decreased. Even then there was, at times, some burning of the grass.

The injury to the grass from the fertilizer was usually greatest where nitrate of soda was used, but even in rims which received only phosphorus some injury was evident. In nearly all cases the injury was temporary, and the grass was quickly replaced by a new and vigorous growth. On any portions where the grass roots were killed

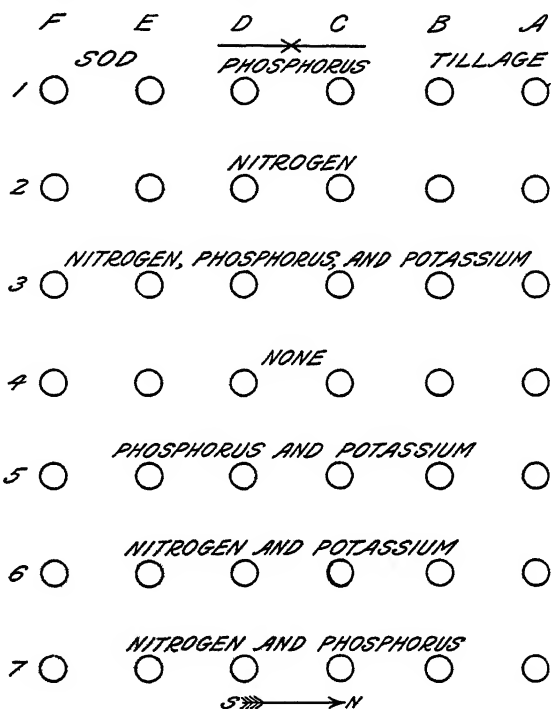


FIGURE 1.—Diagram indicating rims, or cylinders, containing the trees, which were located at the corners of 20-foot squares, the fertilizer treatments used are also shown.

tion for the trees in sod during 1924 was  $17.5 \pm 1.88$ , for those under cultivation,  $27.1 \pm 3.02$ . The coefficient of variability for trunk diameter of all the trees was  $14.3 \pm 1.07$ .

From 1920 through 1923, two to three green-manure crops were grown annually in the rims. Whenever possible, records of height or weight of these covers were taken. These records were reduced to percentages, the rim with the heaviest cover crop each time being rated at 100. Table 2 shows the average percentages of the five cover crops which were measured, each being recorded in percentage of the largest cover of the respective crop. As judged by the growth of these cover crops, the soil in the rims showed a high degree of uniformity.

TABLE 2—Average of five cover crops before fertilization, expressed in percentage of the heaviest cover of each cover crop

Row	Percentage crop cover in row indicated						Average
	F	E	D	C	B	A	
No 1.....	73	60	60	58	52	74	63
No 2.....	81	67	71	69	58	76	70
No 3.....	66	55	62	58	67	90	66
No 4.....	77	65	70	64	58	78	69
No 5.....	81	66	72	79	51	78	71
No 6.....	79	66	68	75	70	62	70
No 7.....	66	57	61	73	63	73	66
Average.....	75	62	66	68	60	76	-----

### FERTILIZER TREATMENTS

Figure 1 shows a diagram of this block with the fertilizer treatments indicated. Table 3 gives the weights of the various salts used and the time of application. All fertilizers were chemically pure materials. It was originally intended to use amounts equivalent to 50 pounds of N, 100 pounds of  $P_2O_5$ , and 50 pounds of  $K_2O$  per acre for trees standing 100 to the acre. The reasons for changes from this plan will be mentioned later.

TABLE 3.—Time of fertilizer applications and quantities used for each rim

Treatment and date applied	Weight (grams) and kind of fertilizer used in row indicated						
	1 (P)	2 (N)	3 (N,P,K)	4 (check)	5 (P,K)	6 (N,K)	7 (N,P)
<b>NaNO<sub>3</sub></b>							
Apr 18, 1925.....		906	906	-----	-----	906	906
May 3, 1926.....		45	45	-----	-----	45	45
June 7, 1926.....		453	453	-----	-----	453	453
June, 1926.....		408	408	-----	-----	408	408
May 5, 1927.....		337	337	-----	-----	337	337
May 18, 1927.....		338	338	-----	-----	338	338
June 10, 1927.....		337	337	-----	-----	337	337
<b>CaH<sub>4</sub>(PO<sub>4</sub>)<sub>2</sub>H<sub>2</sub>O</b>							
Apr 18, 1925.....	534	-----	534	-----	534	-----	534
May 3, 1926.....	267	-----	267	-----	267	-----	267
June 7, 1926.....	267	-----	267	-----	267	-----	267
May 5, 1927.....	534	-----	534	-----	534	-----	534
May 18, 1927.....	267	-----	267	-----	267	-----	267
<b>K<sub>2</sub>SO<sub>4</sub></b>							
Apr 18, 1925.....	-----	-----	293	-----	293	293	-----
May 3, 1926.....	-----	-----	147	-----	147	147	-----
June 7, 1926.....	-----	-----	147	-----	147	147	-----
May 5, 1927.....	-----	-----	293	-----	293	293	-----
May 18, 1927.....	-----	-----	147	-----	147	147	-----

The first application of fertilizers in the rims was made early in 1925. At that time there was a rye cover crop in the cultivated rims. Partly because of the fertilization, and partly from unknown causes, the growth of the rye cover crop in the different rims varied considerably when the crop was ready to be spaded under in late May. To meet this contingency, several beds had been built and filled 1 foot deep with soil similar to the topsoil in the rims. These were sown to rye when the rims were seeded. The rye in each rim was cut and the tops weighed. Enough rye in one of the beds was then cut to make the total weight of the tops in rim and bed 1 kg. The roots of this area in the bed were then dug and all tops and roots spaded into the rim. Thus each rim under cultivation received 1 kg of tops and the roots which went with those tops. No attempt was made to balance the organic matter in the sod rims.

Because of the variations in growth of the cover crops no more were seeded in the rims receiving cultivation. In the fall of 1925, rye was again sown in the beds. On May 20, 1926, this rye was dug, the dirt shaken from the roots, and the tops and roots chopped up together. Two kilograms of this chopped rye were added to each rim and spaded into the soil. The same method was used in 1927. In this way organic matter was kept from being a serious variable in the cultivated rims.

Early in the experiment it was evident that the normal moisture of the restricted soil of the rims would not be sufficient to maintain tree growth. Whenever the soil became too dry as indicated by soil-moisture determinations, from 1 to 2 inches of water were added to each rim. All trees under cultivation received the same application, and all trees growing in sod were treated alike, but the latter received more frequent applications. If there was excess water at any time, it would drain away freely through the crushed limestone under the rims into the well-drained soil below.

These applications of water did not maintain a sufficiently uniform moisture supply to meet the needs of the trees at all times. During a very dry period in the middle of July, 1927, the soil moisture dropped close to the point of physiological wilting. The drought was followed by a period of wet weather. Between 50 and 60 per cent of the apples on all trees cracked during the wet weather. This cracking is characteristic of the Stayman apple under irregular moisture supply. A few fruits cracked after a dry period in August of the same year. If this irregularity in moisture supply influenced the final results, it should have produced the greatest effect on the most vigorous trees. However, at no time was there any indication of wilting of the leaves, nor was there any indication that normal branch elongation was checked.

#### ESCAPE OF ROOTS FROM THE RIMS

The inner four trees in each row were planted in 1922 and the end trees a year later. During 1926 the older trees, with three exceptions, did not make as much total branch elongation as the younger trees, either under sod or in tillage, regardless of fertilizer treatment. It is probable that the restricted volume of soil was slowing up the growth of the older trees, but was still sufficient for the younger trees.

One of the older trees, D-3, growing in sod and receiving the full fertilizer treatment, was considerably more vigorous than any of the

the ground was sodded over with grass grown in a bed which had been filled with the same soil as the top layer in the rims

TABLE 4—Comparative branch elongation of trees in cultivated and in sodded rims, 1924

Row No	Treatment	Odds <sup>a</sup> in favor of cultivated trees	Row No	Treatment	Odds <sup>a</sup> in favor of cultivated trees
1	P	11 to 1	5	P, K	3 to 1
2	N	102 to 1	6	N, K	102 to 1
3	N, P, K	226 to 1	7	N, P	7 to 1
4	None	102 to 1			

<sup>a</sup> The significance of the difference between any two treatments has been estimated by the use of Student's method for interpreting paired experiments (6). Where, as in this table, the comparison is between sod and tillage, the pairing has been A-P, B-E, C-D (Fig. 1). When contrasting fertilizer treatments have been compared, the pairs have been those with similar letters.

Statisticians are agreed that odds of 30 to 1 may be accepted as clear indication that the difference is significant. Significant odds simply mean that the contrasted data differ by an amount too large to be due to chance. One is justified in placing reliance on the interpretation of this significant difference only in proportion to the success in eliminating all other variables except the treatment under study—a condition very difficult to comply with in horticultural research.

Whenever the grass was high enough, it was cut with hand shears and the clippings weighed. Table 5 gives the weights of these cuttings. There were large differences in sod growth under the different treatments.

TABLE 5—Total weights per rim of grass clippings made during 1925, 1926, and 1927

Row	Treatment	Weight (grams) of grass clipped on lettered rim indicated			Average
		F	E	D	
No. 1	P	1,160	850	787	932
No. 2	N	2,860	2,240	3,968	3,021
No. 3	N, P, K	4,870	4,920	3,068	4,283
No. 4	None	670	490	445	535
No. 5	P, K	1,490	750	1,030	1,090
No. 6	N, K	1,970	2,530	1,733	2,073
No. 7	N, P	4,260	5,600	4,215	4,692

The burning of the grass through the use of nitrate of soda has already been described. The application of the same amount of fertilizer to the rims under cultivation modified the clay and affected the physical condition of the soil, making it less friable. This change in condition was sufficiently pronounced to be noted by field men who hoed the rims. The addition of the other fertilizer materials to the nitrogen fertilizer did not modify this effect. The rims under cultivation that did not receive nitrogen remained in excellent condition throughout the test. The addition of the chopped rye described below probably helped to improve the soil structure.

#### COVER CROPS AND WATERING

The oldest field fertilizer experiments in the United States are located on the college farm on soil very similar to that used in the rims. At the end of 40 years Gardner et al. (5, p. [3]), stated: "Phosphoric acid is the first limiting factor in this soil and until this element is supplied, nitrogen and potash give very little increase in yields."

All of the highest 12 trees with respect to leaf weight received nitrogen, either alone or in combination; half of these were in rims receiving cultivation. Experiments in the college orchards since the planting of the trees, in 1908, which are similar in nature to these more carefully controlled tests, failed to show (1, 2) any clear response to nitrogen applications from trees under cultivation until the orchard was at least 15 years old. The very positive nitrogen return in leaf weight during the third season of the fertilizer applications in the case of the rim trees under cultivation shows how much the use of the confining rim hastened root crowding and soil exhaustion. These differences are shown clearly in Figures 2, 3, and 4. Among the trees receiving nitrogen, those under sod had practically as heavy a leaf crop in 1927 as did those under tillage.

TABLE 6 — *Weights of green leaves picked from apple trees, September, 1927*

Row	Treatment	Weight (grams) of leaves from trees in sod				Weight (grams) of leaves from trees in tillage			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	1, 830	998	1, 295	1, 374	2, 169	3, 145	2, 435	2, 583
No 2.....	N.....	3, 460	2, 314	2, 800	2, 858	2, 113	4, 415	4, 060	3, 529
No 3.....	N, P, K.....	2, 620	3, 791	4, 975	3, 795	3, 623	3, 440	4, 690	3, 913
No 4.....	None.....	529	1, 192	1, 220	980	1, 582	1, 855	1, 950	1, 896
No 5.....	P, K.....	1, 450	950	1, 115	1, 172	2, 140	1, 493	2, 600	2, 078
No 6.....	N, K.....	3, 240	2, 397	3, 370	3, 002	2, 795	2, 600	2, 600	2, 665
No 7.....	N, P.....	3, 810	3, 880	4, 256	3, 982	2, 998	4, 260	3, 630	3, 629

Trees receiving phosphorus showed a definite increase in leaf weights in 1927 over trees similarly treated but not receiving that element. (Fig 2 compared to fig 3). There were three treatments of three trees each, both under sod and under tillage, in which phosphorus was checked against no phosphorus (N, P, K, against N, K; N, P, against N, and P against nothing). In the comparison of these treatments by Student's method, the odds that the trees receiving phosphorus had significantly greater leaf weights than those not receiving phosphorus, under sod and under tillage separately, were both about 68 to 1.

Not one of the 12 trees highest in leaf weight in 1927 stood in the row receiving nitrogen and potassium, in fact, the highest ranking tree in this treatment was fourteenth. The odds by Student's method that the trees receiving the N, K treatment had significantly less leaf weight than those in the adjoining N, P treatment are 276 to 1, (fig 3, C6 and D6, compared to fig 4) while the odds that the addition of K to N alone increased the leaf weights are only 3.5 to 1.

TABLE 7 — *Total branch elongation of apple trees during 1925, 1926, and 1927*

Row	Treatment	Length of branches (centimeters) in sod rims indicated				Length of branches (centimeters) in tillage rims indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	6, 249	2, 022	2, 429	3, 567	6, 142	9, 762	6, 677	7, 527
No 2.....	N.....	10, 764	5, 070	8, 321	8, 052	4, 679	12, 003	11, 759	9, 480
No 3.....	N, P, K.....	8, 132	12, 116	18, 883	13, 044	11, 363	11, 822	17, 508	13, 563
No 4.....	None.....	2, 203	1, 780	1, 740	1, 908	4, 080	4, 207	6, 166	4, 818
No 5.....	P, K.....	4, 518	1, 786	2, 369	2, 891	7, 201	3, 192	10, 012	6, 802
No 6.....	N, K.....	8, 386	5, 660	4, 212	7, 753	6, 738	7, 411	7, 900	7, 350
No 7.....	N, P.....	15, 075	13, 995	13, 162	14, 077	8, 568	9, 609	11, 500	9, 892

other trees receiving either this combination or nitrogen and phosphorus, the combinations that gave greatest growth. During the summer of 1927 this tree was again abnormally vigorous, and the growth of two or three others also seemed longer and the leaves greener than on other trees under similar treatments. Because of these variations, it was decided to explore outside the rims to find whether any roots had penetrated the crushed stone in the bottom of the rims and reached the outer soil. Pits were dug in the late summer of 1927, and about half of the bottom edge of rim D-3 was exposed. One root over 2 feet long, probably in its second season of growth, was found, another about 18 inches long, and several small ones. From about one-third of the circumference of rim A-3, in which the tree was a year younger, 25 g of roots were secured. The longest was not more than a foot and a half, and evidently all were of that season's growth. Other rims in each treatment were partly uncovered, in each case some roots, ranging from small fibers to roots several inches long, were found extending into the surrounding soil. This discovery led to the decision to dig out all the trees in the fall of 1927 and to terminate this phase of the experiment.

Did the escape of roots influence the results enough to destroy their value? When all the trees were taken out of the rims in the fall of 1927, studies were made of root distribution, and any roots penetrating beyond the bottom of the rims were noted. Roots, other than a few small fibers, were found outside of only 17 of the rims. In 4 of these rims (B-1, B-2, D-3, F-1) the roots appeared to be in their second season of growth. If the performance of these four trees is judged by their total branch growth during 1927, all seem to show somewhat greater growth than the performance of similar trees would lead one to expect. While the presence of the younger roots outside the rims probably influenced the trees which produced them, there is no consistent evidence that this influence was great enough materially to change the nature of the results.

Two of the four trees with older roots outside the rims (B-2, D-3) received nitrogen. Any added vigor due to the escape of these roots would serve to accentuate the value of nitrogen in the 1927 records. The importance of this element in the experiment had been established with considerable certainty before this time, consequently, while the extent of some of the differences may have been influenced by the escape of the roots, the relative nature of the records would not be materially affected.

#### LEAF WEIGHT AND BRANCH ELONGATION

When the trees were dug out in the fall of 1927, the following records were taken: Weight of fruit, weight of leaves, 1927 branch growth, total weight of tops, diameter of trunks, and total weight of roots. The weights were all of fresh materials. Some samples were preserved for chemical analysis, and dry weights of these were determined. Since two papers (12, 11) have been published in which certain of the chemical analyses made during the progress of this experiment have been reported, this phase of the work is omitted from the present paper.

From September 21 to 30, 1927, all the leaves were picked from the trees and weighed. Table 6 gives the fresh weights.

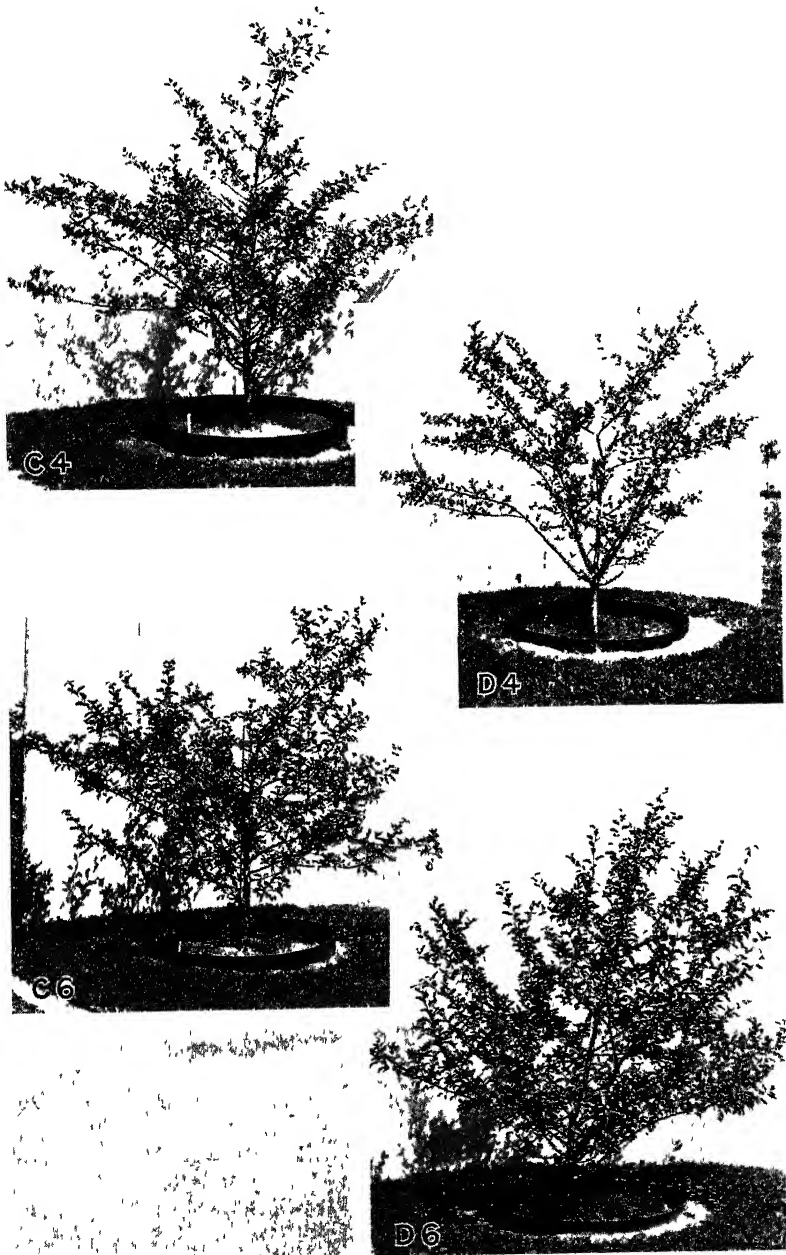


FIGURE 3—Trees receiving no fertilizer under cultivation, C4, and under sod, D4, also trees receiving nitrogen and potassium only under cultivation, C6, and under sod, D6. Photographed September, 1927

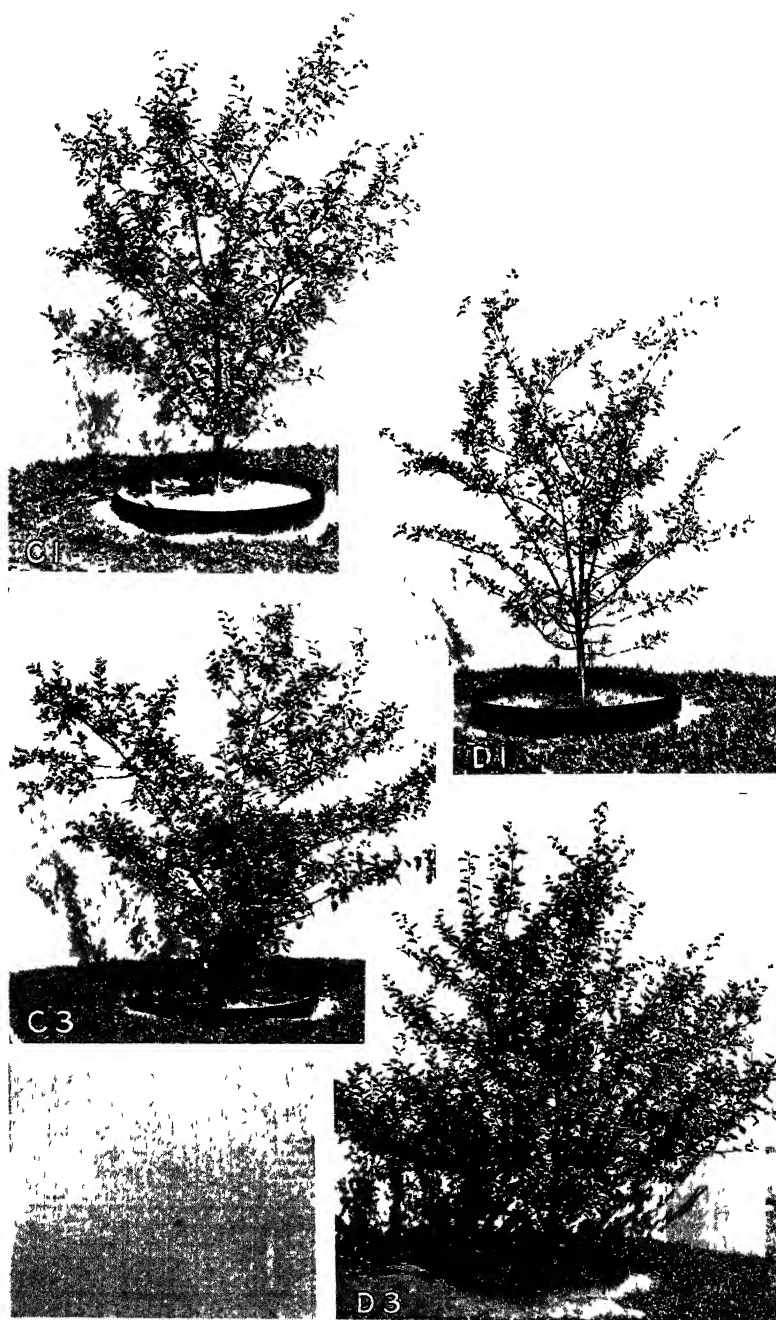


FIGURE 2—Trees receiving phosphorus fertilizer only under cultivation, C1, and under sod, D1, also trees receiving a complete fertilizer under cultivation, C3, and under sod, D3. The roots of D3 escaped from the rim in 1926. Photographed September, 1927.

There is some evidence (Table 8) that the application of phosphorus stimulated tree growth. While the odds are not sufficiently large in all cases, the increase from 1925 to 1927 lends weight to this assumption. There is also some evidence that phosphorus gave a greater response under sod than under tillage.

Table 9 presents the results of treatments that differed only in the addition of potassium. There is no indication that this addition increased branch growth.

TABLE 8—*Influence of phosphorus and potassium on tree growth expressed as odds that gain over contrasted treatment is significant*

PHOSPHORUS				
Relative order of contrasted treatments	Odds for sod and tillage rims		Odds for total growth 1925-27	
	1925	1927	Sod	Tillage
Row 1 (P) greater than row 4 (none).....	* 4-1	47-1	12-1	12-1
Row 3 (N, P, K) greater than row 6 (N, K).....	14-1	340-1	22-1	35-1
Row 5 (P, K) greater than row 4 (none).....	1-1	36-1	41-1	19-1
Row 7 (N, P) greater than row 2 (N).....	3-1	31-1	129-1	2-1
POTASSIUM				
Row 3 (N, P, K) greater than row 7 (N, P).....	2-1	3-1	* 2-1	38-1
Row 5 (P, K) greater than row 1 (P).....	2-1	* 5-1	* 4-1	* 2-1
Row 6 (N, K) greater than row 2 (N).....	1-1	* 16-1	* 2-1	* 6-1

\* Difference reversed

### TRUNK-DIAMETER INCREASE

The extent to which trunk-diameter measurements are used in interpreting orchard experiments makes it desirable to present these measurements for the rim trees. The coefficient of correlation between trunk-diameter increase during the period of fertilization and total branch elongation during the same period is  $0.87 \pm 0.026$ . The correlation between trunk diameter and weight of top is  $0.88 \pm 0.023$ .

A statistical study of percentage increase in trunk diameter from 1924 through 1927, the period of differential treatment, shows that this method of measuring trunk increase is not as satisfactory as the use of the actual gain or the final diameter. Some of the trees that were largest at planting and also at digging showed a smaller percentage increase with the same treatment than some that were much smaller both at planting and digging.

The use of Student's method to group trees of similar age or of the same initial vigor for comparison removes some of the difficulties caused by such variables as those encountered in the study of percentage trunk increase. The method of pairing adopted here grouped trees by age, and to a considerable extent by initial vigor, because of the method of planting (Table 1); but even this has not decreased the degree of variation sufficiently to make it desirable to use percentage increase.

Table 9 gives the trunk-diameter measurements taken when the trees were dug in 1927; Table 10 gives the actual diameter increase from July, 1924, to September, 1927. The very high correlation

As soon as the leaves were harvested, the trees were cut off at a uniform height above the edge of the rims. The 1927 branch growth was measured and the total weight of each top secured. As the branch growth had been measured each year, it was possible to study total branch elongation for the years 1925, 1926, and 1927, during which the fertilizers could have influenced tree vigor. Table 7 gives this record.

Comparing sod and tillage, this table of branch elongation presents practically the same picture as table 6 in which leaf weights are shown, in sod without nitrogen growth decreased to a very pronounced degree,



FIGURE 4.—Trees receiving nitrogen and phosphorus only under cultivation, C7, and under sod, D7. Photographed September, 1927

while the addition of nitrogen gave nearly equal growth under both sod and tillage. The one case among the trees receiving nitrogen that approached nearest to a significant difference between sod and tillage was in the nitrogen and phosphorus treatment, where the odds that sod was better than tillage were 20 to 1.

In order to make a statistical study of the effect of the fertilizers on total branch elongation, the records for each tree for each of the three years were used, thus giving nine measurements when either the three sod or the three tillage trees under one treatment were compared to those under a contrasted treatment. The presence or absence of nitrogen (N *v.* none; N, P, K *v.* P, K) results in such large differences, even with trees under cultivation, that statistical analysis is not necessary.

roots than those not receiving it, among the latter the tilled trees had a larger quantity of roots than the untilled.

The roots from 21 inches down to within 2 or 3 inches of the bottom were mainly of finger size. Many of these were against the rim. Near the crushed limestone the fine feeding roots again became prominent.

Table 12 gives the total weight of the roots for each tree. When the tops of the trees were cut off, each main branch was removed separately and its diameter and weight recorded. Table 13 gives the total of these weights for each tree. Comparing these for root weight and top weight, we find a rather close agreement. All but 2 of the 12 trees highest in root weight are also among the 12 highest in top weight, and all but 2 of the 12 lowest in root weight are among the 12 lowest in top weight. Two trees not receiving nitrogen are among the highest 12 in root weight, and 1 receiving nitrogen is among the lowest 12. One tree of the highest 12 in top weight received no nitrogen; none of the lowest 12 received any nitrogen.

TABLE 11 — *Distribution of apple-tree roots as measured by average weight per tree at different ground levels*

Depth from surface (inches)	Weight (grams) of roots from trees that received—			
	No nitrogen		Nitrogen	
	Sod	Tillage	Sod	Tillage
Crown.....	1,576	2,559	2,741	3,000
0 to 7.....	1,828	2,161	1,067	1,018
7 to 21.....	1,723	4,056	5,390	5,531
Below 21.....	1,569	1,519	2,295	2,672

TABLE 12 — *Total weight of apple-tree roots at digging time*

Row	Treatment	Weight (grams) of roots of trees in sod rim indicated				Weight (grams) of roots of trees in tillage rim indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	6,725	7,895	7,450	7,357	12,275	13,175	11,135	12,195
No 2.....	N.....	11,845	10,366	10,110	10,774	10,300	14,805	13,042	12,716
No 3.....	N, P, K.....	9,420	13,040	16,915	13,125	12,395	13,675	13,315	13,128
No 4.....	Check.....	5,215	9,950	7,350	7,505	10,870	11,790	9,140	10,600
No 5.....	P, K.....	8,195	8,325	6,815	7,778	10,695	7,930	8,615	9,080
No 6.....	N, K.....	9,935	11,090	10,610	10,545	11,700	10,705	10,110	10,838
No 7.....	N, P.....	9,905	11,950	12,730	11,528	13,075	12,260	11,275	12,203

TABLE 13 — *Weight of apple-tree tops at digging time*

Row	Treatment	Weight (grams) of tops of trees in sod rim indicated				Weight (grams) of tops of trees from tillage rim indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	7,745	6,710	9,495	7,983	10,270	16,300	10,410	12,927
No 2.....	N.....	12,960	9,685	12,410	11,685	10,490	21,205	16,875	16,190
No 3.....	N, P, K.....	9,710	13,960	26,910	16,860	13,820	17,215	17,655	16,230
No 4.....	Check.....	4,195	7,510	7,625	6,443	9,745	9,660	9,210	9,538
No 5.....	P, K.....	6,785	7,550	6,790	7,042	10,950	7,230	10,960	9,713
No 6.....	N, K.....	11,330	11,670	14,410	12,470	11,765	11,680	10,570	11,338
No 7.....	N, P.....	13,685	13,980	17,265	14,977	13,455	16,550	13,360	14,455

The significance of gains or losses is more apparent from an inspection of Table 14, which gives the combined top and root weight for each tree. A study of the distribution of the heaviest trees shows that

(0.87 ± 0.026) between trunk measurements and branch elongation would lead one to expect the same conclusions from Tables 9 and 10 as from the tables showing branch growth, and such is the case. The results from the use of nitrogen are nearly the same with sod as with tillage, with the possible exception of the N, P treatment, where the trees growing in sod have the larger diameter. The odds for the significance of this difference in Table 9 are less than 4 to 1; and in Table 10, about 17 to 1; both too small to have much significance. Only one of the first 12 trees in Table 10 did not receive nitrogen, only one of the lowest 12 did receive nitrogen.

TABLE 9—*Trunk diameter of apple trees, September, 1927*

Row	Treatment	Trunk diameter (millimeters) of trees in sod rim indicated				Trunk diameter (millimeters) of tree in tillage rim indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	57	58	58	57.7	69	71	66	68.7
No 2.....	N.....	68	65	71	68.0	66	88	75	76.3
No 3.....	N, P, K.....	68	71	90	77.3	51	77	75	77.7
No 4.....	None.....	41	61	61	54.3	65	65	63	65.3
No 5.....	P, K.....	52	49	60	57.0	65	60	68	64.3
No 6.....	N, K.....	71	69	73	71.0	74	73	70	72.3
No 7.....	N, P.....	68	79	84	77.0	66	77	70	71.0

TABLE 10—*Trunk-diameter increase of apple trees between July, 1924, and September, 1927*

Row	Treatment	Increase of diameter (millimeters) of trees in sod rim indicated				Increase in diameter (millimeters) of trees in tillage rim indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	28	20	20	23	32	28.5	36	32
No 2.....	N.....	38	29	37	35	25	50	44.5	40
No 3.....	N, P, K.....	36	36	41	41	44	49	46	43
No 4.....	None.....	12	21	20	18	27	28	32.5	29
No 5.....	P, K.....	23	20.5	20	21	32	20	42	31
No 6.....	N, K.....	41	30	33	35	35	31	40	35
No 7.....	N, P.....	42	44	45	44	29	30	40.5	33

\* Roots escaped from rims 2 years before digging

#### TOTAL WEIGHT OF TOPS AND ROOTS AT DIGGING

When the trees were dug, the trunk of each was cut off as close as possible to the place where the top had been grafted on the root. The soil was dug in three layers—0 to 7 inches, 7 to 21 inches, and 21 inches to the bottom. Each layer was spread on canvas and the roots separated from the soil and from the crown (Table 11). The trees that received no nitrogen had more roots by weight in the upper 7-inch layer. This layer was filled with very fine roots quite uniformly distributed. In rims receiving similar treatments the weight of the roots in the top layer was approximately the same under sod and under tillage. In the second layer (7 to 21 inches) there were more of the larger roots, and a larger proportion nearer the rims. In this layer the trees receiving nitrogen had many more

By including the six trees receiving common treatment under both sod and tillage in a single plot, as shown in the last column of Table 15, all plots receiving nitrogen alone or in combination made significant gains in total weight of tops and roots over the untreated plot; while phosphorus alone or in combination with potassium failed to give significant increases over the untreated plot

#### RELATIVE VALUE OF PLANT FOODS

There are six cases in Table 16 in which nitrogen, either alone or in combination with other elements, may be compared with non-nitrogenous fertilizers (N *v* P, N *v* P, K, N, P *v* P; N, K *v* P, K, N, P, K *v* P, K, N, P *v* P, K). In every case the gain from the use of nitrogen is significant when the results with sod and tillage are combined

In the three comparisons which differed only by the presence or absence of potassium (P *v* P, K, N *v* N, K, N, P *v* N, P, K), the use of potassium resulted in no significant increase in total weight. When these three treatments were combined, making 18 pairs that differ only in the presence or absence of potash, there was still no significant difference in weight.

In the three comparisons in which phosphorus was the variable (check *v* P, N *v* N, P, N, K *v* N, P, K) it was only when the phosphorus was added to the other two elements that a significant gain was secured. When nitrogen alone was added to each element phosphorus caused a much greater gain than the potassium (N, P *v* N, K). When the three phosphorous treatments were combined in a single comparison, the 18 pairs in sod and tillage together gave odds of 81 to 1 that there was a significant increase in the case of the trees receiving phosphorus.

Past experience has emphasized the importance of studying any fertilizer combination under both sod and tillage separately. When this comparison was made with the trees in the rims, the number of pairs available was reduced from six to three. In general, the use of smaller numbers makes it necessary to obtain larger differences in order to secure the same significance for gains or losses.

When only the three trees were compared, the N, K treatment showed practically significant gains as contrasted with the untreated trees under both sod and tillage; the N, P treatment gave a significant gain over the untreated trees under sod only, and the N, P, K treatment compared to no treatment was significantly different only under tillage. The use of nitrogen alone failed to produce significant increases in either sod or tillage. The high degree of variability and the small number of pairs available caused these decreased odds. This was particularly true in comparisons involving tree D-3, the large weight of which led to such a high standard deviation in this block that the odds were seriously decreased. On the other hand, the trees in the row receiving the N, K treatments were very uniform, and comparisons with this row often gave high odds.

The six cases in which nitrogen was compared to nonnitrogenous fertilizers gave significant increases when the results from sod and tillage were combined; but when these were separated, the only significant increases observed were where nitrogen was used in sod. Even here the use of a complete fertilizer failed to show a significant

all but 1 of the first 12 received nitrogen. The roots of this tree, B-1, probably escaped from the rim in 1926, which may account for the greater growth of this tree. Eight of the highest were in the cultivated half of the area. The twelfth lowest tree received a complete fertilizer; the other 11 lowest trees received no nitrogen. Ten of the lowest 12 were in sod.

The criticism may be made, with justice, that in combining trees of two ages in the same ranking the younger trees may be handicapped. The younger trees planted in 1923 were nearly twice as heavy at planting as those planted in 1922. Their growth was very satisfactory during the preliminary period. There are some indications that the rims checked the growth of the older trees during 1927 to an extent sufficient to permit the younger trees to gain on the older ones, but not to catch up with them, especially in the sod block. None of the sod trees ranking highest in Tables 12 and 13 was in the younger group.

The fact that there are trees of two ages in each treatment does not affect the results when Student's method is used in determining the significance of gains or losses, by this method, trees of the same age standing side by side are compared. Table 15 shows the odds of the significance of the gain of one treatment over another both for the whole row and for those parts in sod and in tillage, based on the total weight of tops and roots.

TABLE 14—Total weights of apple-tree tops and roots at digging time

Rows	Treatment	Weight (grams) of tree tops and roots in sod rims as indicated				Weight (grams) of tree tops and roots in tillage rims as indicated			
		F	E	D	Average	C	B	A	Average
No 1.....	P.....	14,470	14,605	16,945	15,340	22,545	29,475	21,545	24,522
No 2.....	N.....	24,805	20,051	22,520	22,459	20,790	36,010	29,917	28,906
No 3.....	N, P, K.....	19,130	27,000	43,825	29,985	26,215	30,890	30,970	29,353
No 4.....	None.....	9,410	17,460	14,975	13,948	20,615	21,450	18,350	20,138
No 5.....	P, K.....	14,980	15,875	13,605	14,820	21,645	15,160	19,575	18,793
No 6.....	N, K.....	21,265	22,760	25,020	23,015	23,465	22,385	20,680	22,177
No 7.....	N, P.....	23,590	25,930	29,995	26,505	26,530	28,810	24,635	26,658

TABLE 15—Influence of treatment on total weight of apple trees expressed as odds that one treatment is better than another

Relative order of contrasted treatments	Odds for—		
	Tillage rims	Sod rims	Both combined
Row 1 (P) better than row 4 (none).....	13-1	2-1	8-1
Row 2 (N) better than row 4 (none).....	10-1	12-1	98-1
Row 3 (N, P, K) better than row 4 (none).....	43-1	14-1	150-1
Row 4 (None) better than row 5 (P, K).....	2-1	* 1 6-1	1 2-1
Row 4 (N, K) better than row 4 (none).....	28-1	45-1	69-1
Row 7 (N, P) better than row 4 (none).....	11-1	77-1	893-1
Row 1 (P) better than row 5 (P, K).....	5-1	1 6-1	7-1
Row 2 (N) better than row 6 (N, K).....	6-1	* 1 5-1	5-1
Row 3 (N, P, K) better than row 7 (N, P).....	6-1	2-1	6-1
Row 3 (N, P, K) better than row 2 (N).....	* 4-1	6-1	1 7-1
Row 7 (N, P, K) better than row 6 (N, K).....	22-1	4-1	31-1
Row 7 (N, P) better than row 6 (N, K).....	42-1	42-1	1,428-1
Row 3 (N, P, K) better than row 2 (N).....	1 2-1	3-1	4-1
Row 2 (N) better than row 1 (P).....	4-1	41-1	105-1
Row 7 (N, P) better than row 5 (P, K).....	7-1	40-1	74-1
Row 6 (N, K) better than row 5 (P, K).....	6-1	168-1	199-1
Row 3 (N, P, K) better than row 5 (P, K).....	8-1	52-1	142-1
Row 7 (N, P) better than row 5 (P, K).....	23-1	9-1	90-1
Row 7 (N, P) better than row 5 (P, K).....	10-1	50-1	555-1

\* Comparison is reversed.

Table 16 shows that trees under tillage blossomed somewhat earlier and considerably more heavily than trees in sod receiving the same fertilizer treatment. The growth records also show how closely in this instance blossoming was related to tree vigor. It is probable that this growth relation is the cause of the decreased blooming of trees in the sod, and also of the greater bloom on trees receiving nitrogen and phosphorus.

Table 17 gives the total weight of fruit on the trees in 1927. Because of the irregular water supply, most of the fruit dropped to the ground just as it was maturing. The weights given include the drops. The rank in fruit yield is not quite the same as in the number of clusters removed in the spring. Part of the irregularity is due to some rather severe spray burning that injured the clusters which were left. For this reason, and because the record is for a single year only, not much importance should be attached to yields in interpreting the results of this experiment. In general, it may be said that the trees receiving nitrogen and phosphorus and those receiving nitrogen, phosphorus, and potassium bloomed most heavily, but that the trees receiving only nitrogen or nitrogen with potassium made a relatively heavier set of fruit.

TABLE 17 — Total weight of apples picked and dropped from trees, 1927

Row No	Treatment	Weight of fruit (grams) from trees in sod runs indicated				Weight of fruit (grams) from trees in tillage runs indicated			
		F	E	D	Average	C	B	A	Average
1	P	230	0	0	77± 42	590	800	7,240	2,877±1,203
2	N	1,290	5,390	5,305	4,052± 771	1,880	9,060	4,865	5,208±1,148
3	N, P, K	1,230	2,280	7,415	3,642±1,033	910	6,375	5,750	4,345± 952
4	None	0	0	0	0	0	180	320	167± 12
5	P, K	0	0	0	0	3,880	1,710	1,530	2,373± 416
6	N, K	440	9,930	9,650	6,673±1,661	5,220	5,185	2,660	4,355± 467
7	N, P	980	225	4,730	1,978± 768	7,130	2,230	7,883	5,748± 977
					2,346± 605				3,590± 455

### CORRELATIONS AMONG GROWTH AND YIELD RECORDS

There are close correlations among the growth measurements. The treatments which gave the most branch growth also gave the largest trunk diameters and the greatest weights of the trees. Likewise, the individual trees showing the most branch growth also showed the largest trunk diameters and total weights.

Table 18 gives the correlations among the growth measurements studied. In each case the correlation is very high, over 0.8. The high correlation of the various growth measurements with trunk-diameter measurement is especially important because of the frequency with which this measurement is used as an index of growth in orchard experiments. Furthermore, the increase in trunk diameter during the period of fertilization is closely correlated with branch elongation during the same period. A study of the correlations with weight, which must be taken as the absolute measurement of growth, shows that both top weight and total weight are closely correlated with trunk diameter, and top weight is just as closely correlated with branch elongation.

increase over the combination of phosphorus and potassium. This failure, again, was probably due to the abnormal performance of tree D-3

These results are very similar to those secured under field conditions with this same soil (1, 2) In a study of contrasting fertilizer treatments carried on for 21 years in a cultivated orchard it was found that the results from plots treated with phosphorus and potassium were no better than those from untreated plots, but when nitrogen was added to phosphorus alone or to both phosphorus and potassium there was an increase in cover-crop growth, tree growth, and yield It is probable that the better returns from phosphorus alone in the rims as compared to the P, K treatment in the field are due to the higher organic content in the rim soil where either a sod was grown or a considerable quantity of chopped rye added

#### WEIGHT OF FRUIT

As soon as the buds began to unfold in 1926, it was apparent that some of the older trees were carrying a considerable number of blossom clusters These were more numerous on the most vigorous trees Since permitting trees of this age to mature a crop might check their growth to such an extent as to influence the final results, all blossom clusters were removed in the early pink stage Table 16 shows the number removed from each tree

When the buds began to open in 1927, many of the trees were overloaded with bloom, and the blossom clusters were accordingly thinned to a stand approximately 6 inches apart, by pinching out the excess blossom clusters as soon as they were sufficiently expanded to be reached easily Table 16 shows the number of clusters removed It should be remembered that the outer rows of trees were a year younger than the inner four

TABLE 16.—*Blossom clusters removed from apple trees in 1926 and 1927*

#### BLOSSOM CLUSTERS REMOVED, 1926

Row	Treatment	Number of blossoms removed per tree in sod rims indicated			Number of blossoms removed per tree in tillage rims indicated		
		F	E	D	C	B	A
No 1.....	P.....	0	0	0	2	0	11
No 2.....	N.....	0	0	21	17	50	4
No 3.....	N, P, K.....	3	227	98	114	175	45
No 4.....	None.....	0	0	0	0	5	1
No 5.....	P, K.....	0	0	0	10	9	9
No 6.....	N, K.....	0	70	32	7	9	1
No 7.....	N, P.....	4	42	99	87	264	2

#### BLOSSOM CLUSTERS REMOVED TO THIN TO 6 INCHES, 1927

No 1.....	P.....	0	0	0	118	50	132
No 2.....	N.....	0	123	340	113	242	118
No 3.....	N, P, K.....	165	500	577	500	542	395
No 4.....	None.....	0	0	0	0	0	32
No 5.....	P, K.....	0	0	0	200	75	84
No 6.....	N, K.....	0	309	270	335	275	0
No 7.....	N, P.....	173	352	600	722	830	237

Thus it seems that the amount of bearing surface, as indicated by total branch growth, is an important factor in blossom production.

### SUMMARY AND CONCLUSIONS

The use of metal cylinders or rims as a means of studying the effect of different fertilizer treatments on apple trees proved valuable in hastening the fertilizer responses.

The escape of roots from certain rims increased the variability in the growth of the trees, and consequently decreased the accuracy with which the results could be interpreted. However, this decreased accuracy did not destroy the value of the results.

There is a very high correlation—over 0.8—among the growth factors studied, namely, weight of tops, weight of roots, trunk diameter, and branch elongation. The studies of blossom production, yield, and tree growth indicate that blossom production is most highly correlated with total branch elongation and bearing surface, but that, in these trees, yield has a somewhat lower correlation with tree growth. The quantity of fruit produced is only partly dependent on the number of blossoms.

In general, the results from the use of fertilizers confirm the results of previous research in the orchard. Sod, without the addition of nitrogen, checked the growth of the trees very seriously, even when potassium and phosphorus were applied. However, when nitrogen (especially nitrogen in combination with phosphorus) was applied to trees growing in sod, the growth was nearly as good as that of cultivated trees receiving the same fertilizer.

Trees that were cultivated but received no nitrogenous fertilizer were not as vigorous as those that were cultivated and also received nitrogen.

When phosphorus alone was applied to trees growing in sod, the growth was noticeably greater than that of the untreated check. The addition of phosphorus to nitrogen did not produce a definitely better growth than nitrogen alone, but when phosphorus was added to nitrogen and potash, in sod, growth increased.

The addition of potassium to nitrogen or phosphorus or to both did not modify the growth of the trees but may have increased the setting of the fruit.

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TABLE 18—*Correlations of apple-tree growth records*

Measurements correlate 1	Correlation <sup>a</sup>
Trunk diameter with total weight.....	0.87±0.025
Trunk diameter with weight of tops.....	.88±.023
Trunk diameter with total branch elongation.....	.83±.032
Trunk diameter increase, 1924 to 1927, with branch elongation, 1925 to 1927.....	.87±.026
Branch elongation with weight of tops.....	.88±.023

<sup>a</sup> In biometrical studies a correlation factor of over 0.5 is considered certain correlation, provided that the correlation factor is over six times the probable error. If the correlation factor is less than 0.3 or is less than six times the probable error, there is no evident correlation.

Table 19 gives the correlations of yield and blossom records with the growth measurements taken. It is not safe to generalize on the basis of the yields of one year. As the records stand, the correlations of yield with growth measurements are not nearly so high as those of the growth measurements among themselves. Yield is most closely correlated with total weight, even here the correlation is only 0.55, which figure compares unfavorably with correlations of more than 0.8 among the growth records.

TABLE 19—*Correlations of apple-tree yield and blossom records with growth records*

Items correlated	Correlation
Yield with trunk diameter.....	0.48±0.080
Yield with total weight.....	.55±.072
Yield with weight of tops.....	.59±.075
Yield with total branch elongation.....	.45±.083
Yield with branch elongation, 1925 to 1927.....	.38±.089
Yield with branch elongation of 1926.....	.28±.096
Yield of nitrated trees with 1926 branch elongation of nitrated trees.....	.16±.130
Yield with 1927 branch elongation.....	.24±.098
Yield of nitrated trees with 1927 branch elongation of nitrated trees.....	-.047±.137
Yield with blossom clusters removed, 1927.....	.42±.086
Blossom clusters removed, 1927, with total weight.....	.57±.070
Blossom clusters removed, 1927, with weight of tops.....	.56±.071
Blossom clusters removed, 1927, with trunk diameter.....	.58±.069
Blossom clusters removed, 1927, with total branch elongation.....	.62±.064
Blossom clusters removed, 1927, with branch elongation, 1925 to 1927.....	.36±.091

There is little or no evidence of correlation between yield and branch growth of the previous year or of the same year. Since the 1927 yield shows a fair correlation with total branch growth, but not with the growth of 1926 and 1927, the correlation must be with the earlier growth. The roots of those trees that made a good growth during the early years probably had so completely occupied the soil of the rims that by 1926 root crowding had begun to check their growth. The checking of growth of these vigorous trees created the right conditions for fruit production.

There is a rather low correlation between the yield of 1927 and the number of blossom clusters removed in order to thin them to 6 inches. Spray injury probably accounts for some of this low correlation. The thinned clusters were proportional to the actual number of clusters present on the tree. The number of clusters removed gives a fair correlation with total tree weight, which is also the measurement showing the highest correlation with yield. The number of clusters removed shows an even greater correlation with total branch elongation, though not with branch growth during the period of fertilization.

# THE EFFECT OF YEAST AND CASEIN SUPPLEMENTS TO CORN AND SOYBEAN RATIONS WHEN FED TO RATS AND SWINE<sup>1</sup>

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## INTRODUCTION

One of the problems of the corn-producing area has been the inadequate supply of efficient protein concentrates for livestock. Because of an increasing acreage in soybeans (*Soja max*), in the Middle West, this seed has received attention as a possible protein supplement for corn in animal rations. A large number of feeding experiments with farm animals in which soybeans or soybean products were used have demonstrated the feeding value of this concentrate. In general the impression is created that soybeans, at least in the raw condition, have a limited supplementing value in rations for certain classes of livestock.

The analyses of Street and Bailey (7)<sup>2</sup> show the soybean to be high in protein and fat and low in ash and carbohydrates. The protein values found in these analyses ranged from 36.8 to 45.5 per cent.

The high protein content of soybeans led Osborne and Mendel (4) to investigate their biological character. They fed corn gluten and soybean meal to rats and found that when the meal furnished 4.5 per cent protein in combination with 11.4 per cent protein from corn gluten satisfactory growth resulted. They attributed the supplementing action of soybean protein to its content of lysine and tryptophane, which are lacking in corn gluten.

Osborne and Mendel (5) also investigated the soybean as the sole source of protein for the rat and reported that when the diet was complete in other respects it was adequate for normal growth. They found that soybeans furnished a fair supply of "fat soluble vitamin" and an adequate quantity of "water soluble vitamin." However, their data indicated that soybeans were deficient in mineral matter, especially calcium and chlorine. They also noted that soybeans were not readily consumed by rats, presumably because of the disagreeable taste they possessed.

The analyses of Jones and Waterman (3) and Daniels and Nichols (1) show soybean protein to contain a variety of amino acids, some of which are essential for growth. Jones and Waterman reported a tryptophane content of the soybean protein glycine as 1.37 per cent and of lysine as 9.06 per cent.

It appears, then, from chemical and biological analyses that soybeans should be a good supplement for protein-deficient grains in livestock rations.

<sup>1</sup> Received for publication July 22, 1931, issued April, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited p. 274.

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## EXPERIMENTAL RESULTS WITH RATS

## GROWTH ON A PROTEIN-DEFICIENT DIET

Inspection of Figure 1 shows that growth on the basal ration (No. 56) was slow. This subnormal rate of gain was obtained in 13 trials on this ration. In no instance did any group of animals in this series approach satisfactory growth. The average total gain per animal was 63.7 gm for the slowest growing lot and 121.5 gm for the fastest.

Marked variation in individual growth rates occurred, which is an indication of an inadequate diet. A few individuals grew at a fairly rapid rate, although the majority gained slowly. These results show beyond question that the basal ration is inadequate for satisfactory growth of rats. In attempting to determine the cause of this inadequacy the protein moiety of the mixture was studied. Since the introduction of a larger proportion of soybeans in the ration would render it less desirable for swine, because of the tendency of soybeans to form soft pork, this constituent was not increased in these trials. However, in earlier work a quantity of soybeans furnishing an additional 5 per cent of crude protein was added to the basal mixture without favorable results.

Previous work with rats indicated that the ration might be deficient in total protein. It is generally believed that rations carrying less than 14 to 15 per cent of protein are deficient; the experience of the writers, however, does not entirely confirm this view. By feeding corn and casein diets with a protein level of only 13.6 per cent excellent growth was obtained. (Fig. 1, ration 129.) Moreover, a large number of swine rations containing less than 15 per cent protein produced satisfactory results. Nevertheless, it seemed that the basal ration could be improved by the addition of protein. Then, too, there might be some qualitative deficiency in the mixture that could be corrected. Studies were instituted with this in mind.

## GROWTH ON A DIET WITH PROTEIN ADDITIONS

In the earlier work, tankage, meat and bone scrap, and casein were used as supplements. These were found to have a fairly comparable nutritive value, but since casein gave slightly better results it was selected for the work reported here. The casein was incorporated in the corn-soybean-mineral ration in amounts furnishing 2.25 and 5 per cent of crude protein. Table 1 gives the protein content of the rations.

TABLE 1—Composition of experimental rations

Ration No	Yellow corn	Soybeans	Cooked soybeans <sup>a</sup>	Casein	Yeast	Mineral No 6 <sup>b</sup>	Total protein
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
56.....	84.0	14.0	.....	.....	.....	2.0	13.4
127.....	78.3	13.0	.....	6.7	.....	2.0	17.5
125.....	81.4	13.6	.....	3.0	.....	2.0	15.2
129.....	91.3	.....	.....	6.7	.....	2.0	13.6
130.....	95.0	.....	.....	3.0	.....	2.0	11.3
131.....	81.4	13.6	.....	.....	3.0	2.0	14.6
132.....	75.6	12.7	.....	6.7	3.0	2.0	18.7
133.....	78.8	13.2	.....	3.0	3.0	2.0	16.5
135.....	84.0	.....	14.0	.....	.....	2.0	13.4

<sup>a</sup> Cooked in steam until soft, and dried

<sup>b</sup> Mineral mixture No 6 Limestone, 100 parts, special steam bone meal, 100 parts, salt, 10 parts

## EXPERIMENTAL PROCEDURE

Rats and swine were used as experimental animals in this study. The rats were selected from the stock colony at 24 to 30 days of age, at which time they generally weighed between 35 and 45 g. They were quartered in a steam-heated animal house in individual false-bottom wire cages. A modified McCollum feed cup was used, and the food consumed was determined weekly. Two male and two female animals constituted a test group. Each ration was tested on at least three groups. All the rats used in the experiments received daily irradiations from a quartz mercury vapor lamp for 14 minutes.

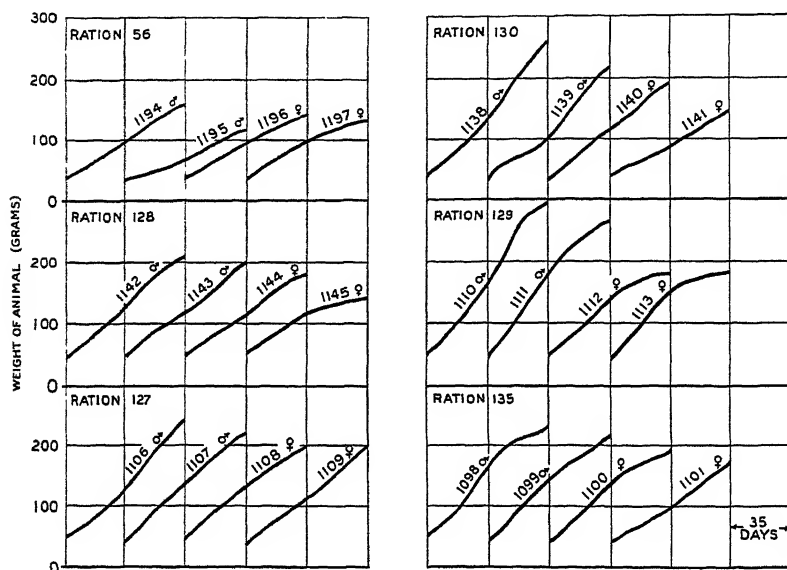


FIGURE 1—Growth of rats on various experimental rations. Slow growth was obtained on the corn-soybean-mineral ration No. 56. Increased growth was apparent on rations 127 and 128, in which 5 and 2.25 per cent casein protein had been added to the basal ration. When the soybeans in rations 127 and 128 were replaced by corn as in Nos. 129 and 130, growth improved still further. Growth was also observed when the soybeans of the basal ration were cooked, as in No. 135. See Table 1 for composition of the rations.

Distilled water was kept before them at all times and was fresh daily. The length of the experimental period was 10 weeks.

The pigs were quartered in dry lots at the experimental swine farm. Each test lot contained 10 pigs of comparable age and weight. The pigs were all either high-grade or purebred Duroc Jersey stock raised on the swine farm. These animals were weighed at 10-day intervals. Food consumption records were obtained from each lot. The experimental period was 70 days.

The basal ration (ration 56) had the following composition: Yellow corn, 84 per cent; soybeans,<sup>3</sup> 14 per cent; salt mixture,<sup>4</sup> 2 per cent.

The experimental rations differ from No. 56 by the substitution of protein concentrates and vitamin B carriers for a part of the corn-soybean mixture. In some trials the soybeans were cooked and in others they were left entirely out of the mixture.

<sup>3</sup> Manchou variety

<sup>4</sup> Composed of limestone 100 parts, special steam bone meal 100 parts, salt 10 parts.

incorporated in the feed in the same ratio as the unprocessed beans. The results are presented in Figure 1 (Ration 135.)

Ration 135, containing cooked soybeans, has a crude protein content of 13.4 per cent. For comparative purposes reference may be made to ration 129, containing approximately the same protein content derived from corn and casein.

Growth on the cooked soybean ration was definitely superior to that obtained with raw soybeans. Moreover, it compared favorably with that obtained by feeding a ration of corn and casein of about the same protein level, although the latter combination was somewhat superior. These results point to the conclusion that soybean protein is not deficient from a qualitative standpoint.

Investigations are being made to determine the cause of the improvement in the nutritive value of soybeans brought about by heating. It is unlikely that any of the major food constituents are changed in a qualitative manner. In an earlier paper<sup>6</sup> it was shown that the digestibility of the ration is not changed by cooking. Although the explanation of the greater nutritive value of cooked soybeans is not clear at this time, there is some reason for believing that certain materials of a toxic nature are removed or destroyed during the heating process.

#### GROWTH ON A DIET WITH YEAST ADDITIONS

In earlier trials the writers tried to improve growth by the addition of dried brewers' yeast to the ration on the assumption that it was deficient in the vitamin B complex, especially in the growth-promoting factor. The results of this study were so varied as to preclude the drawing of any definite conclusions. Osborne and Mendel (5) found soybeans to be an adequate source of "the water soluble vitamin." So far as the writers are aware, no experiments have been reported that demonstrate the relative amounts of vitamin B ( $B_1$ ) and vitamin G ( $B_2$ ) in soybeans. The writers have found<sup>7</sup> that a purified ration, complete except for vitamin B, must contain at least 12 per cent of soybeans in order to produce small weekly gains in the weight of rats. Since corn is rich in the antineuritic vitamin B ( $B_1$ ) and poor in the growth-promoting factor ( $B_2$ ), (2) there is no reason to believe that the ration used by the writers is deficient in the antineuritic vitamin, although it might be deficient in the growth-promoting factor. The work herein deals with the addition of 3 per cent yeast to the basal mixture, and the same supplemented with 2.25 and 5 per cent protein from casein. The results are presented in Figure 2.

The addition of yeast to the basal mixture consistently caused a slight but definite improvement in growth (Ration 131.) However, the improvement was not great enough to be considered satisfactory. From the standpoint of improving corn-soybean rations it was concluded that yeast is of little, if any, value.

When yeast was added to rations containing casein the results were again irregular, confirming results obtained by the writers in earlier experiments.

<sup>6</sup> SHREWSBURY, C. L. THE NUTRITIVE VALUE OF SOYBEANS. THE EFFECT OF HEAT. Paper read before the Amer. Chem. Soc. Annual Meeting, Indianapolis, 1931.

<sup>7</sup> Unpublished data.

Reference to Figure 1 (rations 127 and 128) shows a marked increase in rate of gain when either the high or the low level of casein was employed. Since more favorable growth resulted from the use of the higher level of casein it would appear that, in part, the quantity of protein was an important factor.

The question arose as to whether the increased growth exhibited by the animals on the lower level of casein was due to a true supplementing action of casein for soybean protein, or to a building up of the total protein content of the ration, or to other factors. In order to determine this point animals were placed on a ration of corn and mineral, with casein furnishing 2.25 per cent protein (Ration 130). Here the soybeans of ration 128 were replaced by an equal amount of corn. If the favorable response that was obtained by the use of ration 128 (composed of corn, soybeans, casein, and mineral) was due to a supplementing action of casein for soybeans, then growth on the modified ration (No. 130), in which soybeans were replaced by corn, should be at a slower rate. The results of this trial, presented in Figure 1 (rations 128 and 130), show that the growth of the animals was as good when soybeans were omitted as when they were present, and in some instances even better. The same results were obtained when soybeans were replaced by an equal amount of corn in the ration containing the higher level of casein protein (Ration 129.)

There is no evidence from these experiments that soybean protein was supplemented by the addition of casein. It appears that the protein of soybeans is not utilized above a certain point, owing possibly to factors contained in the soybean, the nature of which is not known. That is, when casein was added to the basal corn-soybean mixture better growth resulted probably not because the ration was qualitatively or quantitatively different from the original ration but rather because the nutrients of casein were utilized readily to supply the deficiencies of corn, whereas those of soybeans were not utilized.

Although casein supplements corn alone, it is indicated from these experiments that in so far as improving the utilization of soybeans in combination with corn is concerned, the addition of casein protein, as a supplement, is of questionable value.

#### GROWTH ON DIETS CONTAINING COOKED OR UNCOOKED SOYBEANS

Since the work did not reveal a qualitative inadequacy of soybeans but rather an inability of the animal organism to utilize the potential nutritive value in them, it was decided to process the beans in several ways before feeding. The work described is concerned with heating, especially cooking.

Osborne and Mendel (5) in 1917 showed that cooking improves the feeding value of soybeans for rats. Later, Robison (6) fed cooked undried soybeans to hogs as a supplement to corn with very satisfactory results. It seemed desirable to study the effect of cooking in more detail.

Soybeans were cooked by passing steam through them until they became soft. Then they were rapidly dried to approximately their original moisture content.<sup>5</sup> Soybeans prepared in this manner were

<sup>5</sup> Cooking and drying were done by the agricultural engineering department.

times that of pigs fed uncooked soybeans, they required only about half as much of feed to produce a unit of gain.

TABLE 2—Data obtained when the experimental rations were fed to swine

[Ten pigs in each lot]

Lot No	Ration No <sup>a</sup>	Average daily feed consumption	Feed required to make 100 pounds gain	Average daily gain	Average gain
		Pounds	Pounds	Pounds	Pounds <sup>c</sup>
1.....	56	2 12	656 3	0 31	27 8
2.....	127	2 77	402 4	69	62 0
3.....	128	2 61	414 6	67	56 6
4.....	129	4 16	344 8	1 21	108 5
5.....	130	3 01	476 4	63	56 9
6.....	131	2 35	550 0	43	38 4
7.....	132	3 46	385 2	1 03	93 0
8.....	133	2 73	373 2	73	67 8
10.....	135	3 45	345 3	1 00	89 9

<sup>a</sup> See Table 1 for composition of rations

When casein protein was added (lots 2 and 3) the gains were greater than on the unsupplemented ration. When corn replaced soybeans in the rations containing casein (lots 4 and 5) the gains were as good as or better than those obtained with soybeans in the ration (lots 2 and 3), comparing lots 4 and 2, and 5 and 3. Dried yeast (lot 6) did not improve the basal ration materially, and yeast additions to rations carrying high and low levels of casein (lots 7 and 8) produced variable results.

In general, there was a high degree of correlation between the data obtained for swine and those obtained for rats.

### CONCLUSIONS

A basal ration of corn, soybeans, and mineral is not adequate for satisfactory growth of rats under experimental conditions or for young pigs in dry lot.

The growth of pigs and rats on corn-soybean rations can be improved by the addition of casein in amounts sufficient to 2.25 or 5 per cent protein.

Although casein supplements corn alone, the addition of casein to corn-soybean rations was of no value in improving the utilization of soybeans. No evidence was obtained that casein protein supplemented soybean protein.

Cooked soybeans have a definitely superior nutritive value to raw soybeans, due to factors not apparent from these experiments. When cooked soybeans were used less feed was required to produce a unit gain in weight than when raw soybeans were used.

The protein of cooked soybeans appears to have a nutritive value somewhat less than an equivalent amount of casein when combined with corn.

The addition of 3 per cent dried yeast to rations of corn and soybeans did not improve the growth rate of the animals sufficiently to make it of economic value.

## EXPERIMENTAL RESULTS WITH SWINE

All of the rations described above were fed to swine. Table 2 gives the data obtained

The pigs weighed about 32 pounds at the beginning of the experiment. In earlier experiments heavier pigs were employed, but it was felt that more satisfactory information could be obtained in this study by the use of smaller animals. One of the writers<sup>8</sup> (8) has demonstrated that 100 to 110 pound pigs thrive on corn-soybean rations in dry lot. Lighter pigs will grow well on corn-soybean

rations if allowed to forage legume pasture, but these lighter animals do not make satisfactory gains if fed in dry lot.

Table 2 shows that the results obtained with swine were substantially the same as those described above for rats. The most striking feature of this experiment was the behavior of the lots on the basal corn-soybean-mineral ration (lot 1) and that on the same ration prepared with cooked soybeans (lot 10).

The pigs fed the basal ration grew very slowly (0.31 pound per day). These animals were all in good condition at the beginning of the experiment, but in a few weeks they became very unthrifty. The variability among individuals was pronounced. Some gained fairly well but the majority remained almost stationary, and a few lost weight. The hair coat in this lot was poor and shaggy, and the animals were humped and thin. However, when cooked soybeans

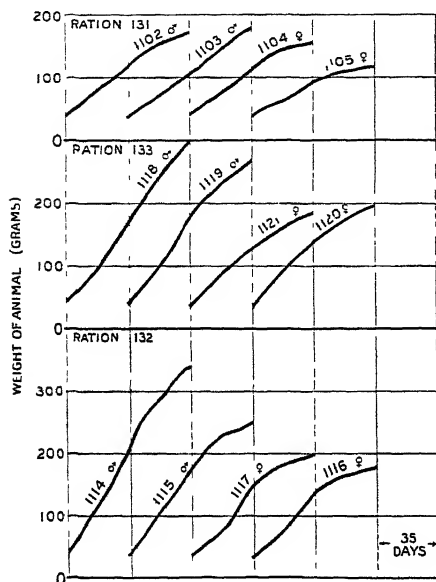


FIGURE 2—Growth of rats on experimental rations to which yeast was added. Growth on ration 131 shows the effect of adding 3 per cent dried brewers' yeast to the basal diet, results should be compared with those for ration 56, Figure 1. Growth on rations 132 and 133 shows the effect of supplementing the basal diet with 3 per cent dried brewers' yeast and with 5 per cent casein protein in the case of 132, and 2.25 per cent in the case of 133, results should be compared with those for rations 127 and 128, Figure 1.

replaced uncooked soybeans the situation was reversed. The pigs made an average daily gain of 1 pound and all were fat and had good coats of hair. Those that were smallest at the start grew rapidly and remained thrifty throughout the feeding period, while the smaller pigs receiving the raw soybean ration remained small and their condition became poorer as the experiment proceeded.

While too much emphasis should not be placed on feed consumption in the case of these pigs, because of the tendency of all the groups to waste feed at the self-feeders, it is interesting to note the greater utilization of feed by the pigs on the ration containing cooked soybeans. Although they made an average daily gain of more than three

<sup>8</sup> VESTAL, C. M., SOYBEANS FOR HOGS, Ind. Agr. Expt. Sta. Hog Summary Leaflet H-8. 1924. [Mimeographed.]

# THE EFFECT OF ENVIRONMENT ON THE NEMATODE OF THE TOMATO GALL<sup>1</sup>

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## INTRODUCTION

Root knot has been known to occur in the United States since 1805. Neal (11, p. 9)<sup>3</sup> stated that he found it prevalent in Florida in 1876 and that "from time immemorial [it] had been dreaded as a foe to gardens and groves." The range of the disease now extends from the Atlantic coast to the Pacific. The use of the glass house in plant industry has brought the climate of the South to the Northern States, and wherever this artificial climate is maintained root knot eventually occurs.

The causal organism, a nematode (*Heterodera radiculicola* (Greeff) Müll.),<sup>4</sup> has been extensively studied in this country by Neal (11), Atkinson (1, 2), Stone and Smith (14), Bessey (3), Godfrey and Morita (6), and Newhall (12).

The purpose of the present paper is to add to the general knowledge of the activity of the root-knot organism within the gall as influenced by temperature and moisture combined, moisture alone, desiccation, and decay of attached and detached galls.

## NATURE OF THE TOMATO GALL AND ITS NEMATODE POPULATION

Nematode galls caused by *Heterodera radiculicola* vary in size and form according to the number and position of female nematodes present in them. On the root system of a single badly infected tomato plant the galls may range in size from one-sixteenth of an inch to 1 inch in diameter and may be 3 inches long. The material used in the present investigation was obtained from commercial greenhouses in which tomatoes, cucumbers, and lettuce were grown.

Tomato galls have a large preponderance of cortical cells. An examination of cross and longitudinal sections shows that the pressure of these cells has altered the direction of the conducting channels of the root system, even to the breaking point. Atkinson (1), Stone and Smith (14), and Bessey (3), who have made particular studies of such distortions, ascribe a considerable portion of nematode injury to this disturbance, and a small amount of injury to the feeding habits of the parasite.

If a large nematode gall from a tomato plant is kept in a moist chamber for a few days the nematode population can be more easily examined. When the gall is broken open a hand lens will reveal a great many female nematodes, which appear as small, more or less

<sup>1</sup> Received for publication July 6, 1931 issued April, 1932. Published as contribution No. 122 of the Massachusetts Agricultural Experiment Station.

<sup>2</sup> The author acknowledges his indebtedness to Prof. A. Vincent Osmun for his untiring interest in the progress of this investigation.

<sup>3</sup> Reference is made by number (italic) to Literature Cited p. 284.

<sup>4</sup> *Caconema* is the genus proposed by Cobb as reported by Stiles (15, p. 118-121) in 1924. Because the root-knot organism differs in some respects from the true *Heterodera* as represented by the sugar-beet nematode (*Heterodera schachtii* Schmidt), Cobb believes the genera should be separated. *H. radiculicola* (Greeff) Müll. has, however, been the accepted name since 1884 (10). In 1872 Greeff (7) called the root-knot nematode *Anguillula radiculicola* and in 1889 Neal (11, p. 27) called it *A. arenaria*.

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Glass tumblers were employed as containers. Each contained 200 gm of air-dried soil brought up to 60 per cent moisture content. Inoculations were made by mixing 5 g of chopped nematode galls with the soil in each tumbler. For three weeks after the inoculations the tumblers were allowed to remain at greenhouse temperature. Then the soil in each was thoroughly mixed, adjusted to a specified moisture content, planted with two tomato seedlings, and placed in the temperature-control apparatus with the soil level below the level of the water bath. Table 1 gives the ranges of temperature and moisture. The experiments were allowed to run 30 days, during which time the moisture was adjusted daily by adding water equal to the loss in weight. The 30-day period was chosen because it was believed that in that time galls would have formed but probably would not have interfered seriously with the general character of the root system. However, certain discrepancies appear in Table 1 that may be due to unrecognized nematode activity. At the conclusion of the experiment, the soil was carefully washed from the roots of the plants and the better plant in each container was placed in series A and the poorer plant in series B. By the use of the scoring method suggested by Free (4), the root systems were compared in a manner that gave a definite score for each system within the series. Experiment 1 was conducted during January, 1926, experiment 2 was carried on from June 23 to July 24, 1926, and therefore had the better light of mid-summer. The scores showing the relative effects of temperature and moisture combined are presented in Table 1.

TABLE 1—*Effect of temperature and moisture on the ratio of galls to root systems*

[The larger values indicate proportionately fewer galls] \*

Temperature (° C )	Moisture content	Ratio of galls to root system				Distribution of galls
		Experiment No 1		Experiment No 2		
		Series A	Series B	Series A	Series B	
	<i>Per cent</i>	<i>Relative value</i>	<i>Relative value</i>	<i>Relative value</i>	<i>Relative value</i>	
15.....	40	65	59	50	14	Galls on main and lateral roots
	60	43	48	10	41	Do
	80	43	37	59	41	Galls on lateral roots
	100			99	99	Few small galls on lateral roots
18.....	40	59	71	5	14	Galls on main and lateral roots
	60	43	48	32	55	Do
	80	94	94	72	72	Galls on lateral roots
	100			99	99	Small galls on lateral roots
21.....	40	54	48	32	41	Galls on main and lateral roots.
	60	94	77	41	32	Do
	80	99	94	64	72	Galls on lateral roots
	100			99	99	Small galls on lateral roots
24.....	40	6	12	14	19	Galls on main and lateral roots
	60	37	31	46	50	Do
	80	83	88	59	19	Do
	100			90	90	Small galls on lateral roots
27.....	40	1	1	19	14	Galls on main and lateral roots
	60	25	25	32	50	Do
	80	83	83	77	2	Galls on lateral roots.
	100			86	81	Do
30.....	40	12	6	1	1	Galls on main and lateral roots
	60	19	19	64	64	Do
	80	71	77	23	46	Do
	100			81	86	Small galls on lateral roots

\* The values are relative only within the series under which they occur. The better plants are in series A, the poorer in series B, experiment No 1 was conducted in January, No. 2 in June and July.

opalescent, pearlsh bodies On some of these bodies there is a brownish area which, when examined under the low-power lens of the microscope, is seen to be a mass of eggs containing nematode embryos in all stages of development Dark areas in the tissue of the gall, when examined with the low-power lens, show a mass of nematodes in all stages, ranging from the undifferentiated granular egg to a female nematode in the process of enlarging to the pyriform shape that characterizes maturity Galls in the last stages of decay actually teem with nematode life

Among the numerous forms of the root-knot nematode in a single tomato gall, there appears infrequently a mature female that corresponds to the brown cyst stage of the sugarbeet nematode, *Heterodera schachtii* It contains a mass of eggs Specimens of this form were identified by G Steiner, senior nematologist of the United States Department of Agriculture, as females of the root-knot nematode The existence of the cyst form has been denied by some investigators, who have made use of its apparent nonexistence in separating the two species *H radicola* and *H schachtii* Not in recent years has the subject of the cyst stage of *H radicola* appeared in print, yet the subject exists in a controversial state in previous literature on the subject, the cyst form being both described and denied

It is important to state that the cyst form was found in galls from mature greenhouse plants growing in a soil that was fairly dry—so dry that the root system when pulled and shaken was left with scarcely any soil clinging to the roots The galls were large, one-half to 1 inch in diameter, and more woody or corky than is common

Examinations of cucumber and lettuce galls have failed to reveal the cyst form Furthermore, the average gall on the tomato plant does not carry it Dry soil conditions seem to be the determining factor where cyst-bearing galls are found.

#### COMBINED EFFECT OF TEMPERATURE AND MOISTURE ON NEMATODES

Observations in greenhouses where root knot was seriously interfering with the productivity of the tomato crop indicated that the degree of infestation might be influenced by a soil-moisture relationship. In houses facing the south, the soil, as a rule, is moister on the north half Usually it is necessary to water more frequently on the south half, especially the spring crop The examination of tomato roots in such houses showed a general infestation of nematodes, but the plants from the north half were less galled than those in the drier soil of the south half Plants near a leaky water valve and a dripping pipe from an overhead watering system also showed fewer and smaller galls As a result of these observations a series of experiments designed to yield information on the combined effect of temperature and moisture on root knot was carried out.

The range of soil temperatures employed in these experiments, 15° to 30° C., was between the minimum and maximum that would permit gall formation, and the range of moistures, 40 to 100 per cent of the moisture-holding capacity of the soil, was such as would support tomato growth. The temperatures were maintained by the constant soil-temperature apparatus. The saturated soils produced plants that compared favorably with the plants at other soil moistures.

EFFECT OF MOISTURE ON NEMATODES

In order to obtain more definite information concerning the effect of moisture on nematodes in galls, fresh tomato galls as large as one-half inch in diameter were mixed with 400 g. of air-dry soil and kept in pint glass jars. To the soil was added sufficient water to produce the range of soil moistures shown in Table 2. The jars were loosely capped with paper, which was held in place by rubber bands. This helped to maintain a constant soil moisture. The jars were weighed every 48 hours and brought up to weight when necessary. At the end of the 31-day period the soils were mixed with enough nematode-free soil (steamed soil) to fill a 6-inch pot. The pots were then planted with tomato seed and four weeks later the seedlings were examined, with a final examination 15 weeks after the seed was planted. The results, which are presented in Table 2, show that the nematodes survived at all moistures except at 0 per cent, i. e., in the air-dry soil. Even in soils too deficient in moisture (10 and 30 per cent) to sustain crop plants, the nematodes were able to survive.

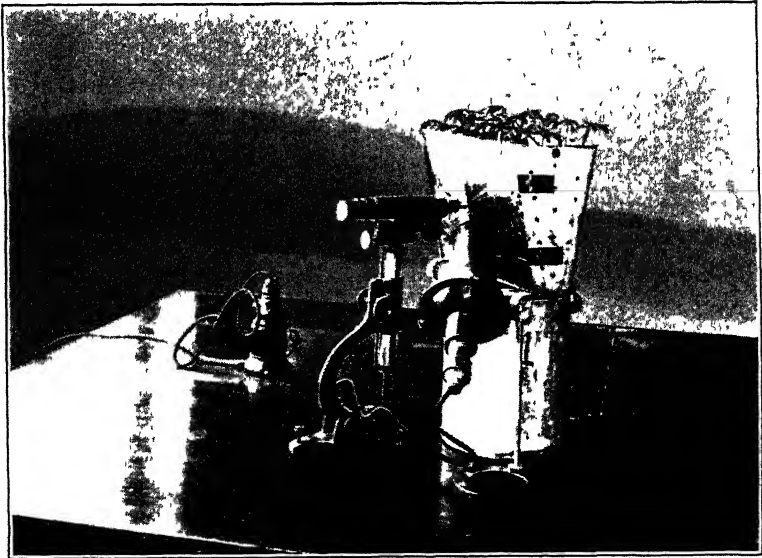


FIGURE 1—Apparatus used for observing root invasion by nematodes

TABLE 2—*Effect of soil moisture upon the survival of the nematode in galls*

Moisture in soil (per cent) <sup>c</sup>	First examina- tion <sup>a</sup>		Second examina- tion <sup>b</sup>		Moisture in soil (per cent) <sup>c</sup>	First examina- tion <sup>a</sup>		Second examina- tion <sup>b</sup>	
	Plants nema- tode free	Plants nema- tode infected	Plants nema- tode free	Plants nema- tode infected		Plants nema- tode free	Plants nema- tode infected	Plants nema- tode free	Plants nema- tode infected
	Num- ber	Num- ber	Num- ber	Num- ber		Num- ber	Num- ber	Num- ber	Num- ber
0.....	5	0	3	0	70.....	0	5	0	3
10.....	0	5	1	2	80.....	0	5	0	3
30.....	2	3	0	3	90.....	0	5	0	3
50.....	3	2	0	3	100.....	0	5	1	2
60.....	1	4	0	3					

<sup>a</sup> 5 plants examined 4 weeks after seeding  
<sup>b</sup> 3 plants examined 15 weeks after seeding.

<sup>c</sup> Percentage of saturation.

Within the range of soil temperatures employed, it is apparent that there were more galls in proportion to the size of the root system at the higher temperatures, 24°, 27°, and 30° C, with no particular temperature of critical importance. At lower temperatures, 15°, 18°, and 21°, the root systems were freely galled, but much less so than those at the higher temperatures. As the optimum soil temperature for the growth of tomatoes is close to 25° (5, 9), it is to be expected that root knot will be prevalent on tomato at about that temperature. The relative values in Table 1 do not change markedly with a slight increase in temperature. This finding is in agreement with that of Godfrey (5) who, in addition to tomato, used cucumber, tobacco, and soybeans as indicator plants. All these plants have a fairly high optimum soil temperature.

The differences in the results obtained with the different soil moistures were quite marked. Plants grown in the saturated soils (100 per cent moisture) were characterized by long slender roots burdened with very few galls. In such soils the galls were more plentiful at the higher temperatures. No plant was free of galls. In general, as the moisture content of the soil was increased, the proportion of galls decreased. There were more galls at the 60 and 80 per cent soil moistures than at the 40 per cent moisture, but the root systems of the plants at 40 per cent were so small that the plants would soon have died. The plants at 100 per cent soil moisture showed signs of spindling, but this was due to too much moisture and not to the presence of nematodes.

An examination of the data in Table 1 showing the distribution of galls on the roots indicates that the main root of the tomato seedling becomes burdened with galls at the lower moisture contents and also at the higher temperatures. At the higher moisture contents the galls are, for the most part, on the side roots. Infection on the main root interferes more seriously with the growth of the plant than does the presence of galls on the lateral roots.

As previously stated, in the soils of highest moisture content (100 per cent) the roots were very long and slender and such roots had few galls, if any. It therefore appeared possible that the size of the root might aid or prevent invasion by the nematode. It is commonly understood that the invading nematode attacks the loosely massed cells of the meristematic tissue of the root tip, and so gains entrance to the root. By employing a root cage with tomatoes growing in a nematode-infested soil, a horizontal microscope, and a strong light focused on root tips, the writer was able to make direct observations of a nematode in the process of invading a root. The worm had worked its way in at the root tip and upward through the meristematic region, the undulations of its body aiding in the observation. A small portion of the posterior end had apparently not fully entered the root tip, for it was in the place normally occupied by the root cap. A measurement with an ocular micrometer determined the length of the organism in the observed stage as 933 $\mu$ . (Fig. 1.)

Beaded arrangements of small galls seem to confirm a theory that the nematode does not stop after forcing its way into the root tip, but travels into the tissue that is developing the central cylinder to avoid being carried forward by the progressive growing point of the root tip.

negative results. Unfortunately, it was not anticipated that eradication might be secured in less than two weeks, and observations were unduly delayed. All remaining groups gave negative results, as shown in Table 4.

TABLE 4.—*Effect of desiccation in soil upon the survival of nematodes in galls*

Period in air-dry soil (weeks)	Experiment No. 1, Dec 10, 1926– Apr 23, 1927					Experiment No. 2, Aug 23– Dec 16, 1927				
	Nematode galls * in pot No —					Nematode galls * in pot No —				
	1	2	3	4	5	1	2	3	4	5
Check.....	+	+	+	—	+	+	+	+	+	—
1 $\frac{1}{2}$ .....	—	—	—	—	—	+	+	+	+	—
1.....	—	—	—	—	—	+	+	+	+	—
1 $\frac{1}{2}$ .....	—	—	—	—	—	0	0	+	+	—
2.....	0	0	0	0	0	0	0	0	0	0
2 $\frac{1}{2}$ .....	0	0	0	0	0	0	0	0	0	0
3.....	0	0	0	0	0	0	0	0	0	0
3 $\frac{1}{2}$ .....	0	0	0	0	0	0	0	0	0	0
4.....	0	0	0	0	0	0	0	0	0	0
4 $\frac{1}{2}$ .....	0	0	0	0	0	0	0	0	0	0
5.....	0	0	0	0	0	0	0	0	0	0
5 $\frac{1}{2}$ .....	0	0	0	0	0	—	—	—	—	—

\* The sign (+) indicates the presence of nematode galls, the dash (—) infers that no examination was made for the indicated period, and 0 indicates the absence of nematodes.

The experiment was repeated with galls of the  $\frac{1}{2}$ -inch size and provision made to reduce all periods to multiples of  $\frac{1}{2}$ -week duration. In the second experiment, eradication was effected in more than one and one-half weeks and less than two.

It is apparent that the  $\frac{3}{4}$ -inch galls used in the preliminary experiment required a longer period of desiccation than the  $\frac{1}{2}$ -inch galls employed in the two experiments shown in Table 4. From these results it is obvious that the length of the survival period is influenced by the size of the galls as well as by the length of time that the galls remain in the air-dry soil.

#### DECAY OF GALLS, ATTACHED AND DETACHED, IN SOILS OF DIFFERENT MOISTURE CONTENT

A study of the influence of environment on the nematode organism is more involved under natural conditions than it is in the laboratory. Particularly is this true when the galls are large.

In addition to harboring a dormant phase of nematode activity, the galls certainly must act as protective agents to their nematode population. A decayed gall has less protective action than a fresh one. A gall attached to a living root may persist in the fresh stage longer than does a detached gall. Even though these galls remain in the soil for a considerable time, they may resist decay owing to the antiseptic properties of living cells, which are kept alive by the root of which they are a part. That such a relation exists is borne out by the following experiment in which the decay of galls, attached and detached, was studied in soils holding different percentages of moisture.

Equal weights (10 pounds) of air-dry soil were placed in containers divided into five series of five pots each at moisture contents of 0, 40, 60, 80, and 100 per cent of the water-retaining capacity of the

## EFFECT ON NEMATODES OF FLOODING A GALL-INFESTED SOIL

After it had been determined that a saturated soil (100 per cent moisture) will support nematode life and allow invasion to take place, it was decided to carry the study of moisture relationships one step further and include a flooded soil.

For this study galls were buried in sand in glass tumblers and the water level maintained at least one-half inch above the surface of the sand for various periods, as shown in Table 3. At the end of each period a tumbler was removed and its contents mixed with sterile soil in a 6-inch pot, which was then planted with tomato seed. A pot containing sterile soil was likewise planted with tomato seed and placed in the group of pots as a check on contamination. At the close of the experiment no galls were found in the check pot, but galls were present in all other pots. It is therefore apparent that even 28 days of continuous flooding is not sufficient to destroy the nematodes in gall-infested soil.

TABLE 3—*Effect of flooding upon the survival of nematode in galls*

Period flooded (days)	Days from seed	First examination		Final examination		Period flooded (days)	Days from seed	First examination		Final examination	
		Plants nematode free	Plants nematode infected	Plants nematode free	Plants nematode infected			Plants nematode free	Plants nematode infected	Plants nematode free	Plants nematode infected
		Number	Number	Number	Number			Number	Number	Number	Number
0.....	25	4	6	0	3	6.....	19	0	10	0	3
1.....	24	2	3	0	3	7.....	18	0	10	0	3
2.....	23	0	10	0	3	10.....	25	0	10	0	3
3.....	22	0	10	0	3	14.....	24	0	4	0	3
4.....	21	0	10	0	3	21.....	42	7	19	0	3
5.....	20	0	10	0	3	28.....	35	20	9	0	3
Check....	35	20	0	3	0						

## EFFECT ON NEMATODES OF DESICCATION IN SOIL

Although several investigators have reported that nematodes can be destroyed by the process of gradual desiccation, no information was available as to just what is the maximum period that large galls will support nematode life in the desiccating atmosphere of an air-dry soil. Accordingly, a series of experiments designed to throw light on this point was conducted.

In a preliminary experiment, large galls (about three-fourths inch in diameter) were introduced into 6-inch pots of air-dry soil. After 2, 3, and 4 weeks these pots, in replicates of five, were moistened and seeded with tomato. The 3-week and 4-week desiccation periods destroyed all nematode life, but the 2-week period did not.

For the next two experiments galls one-half inch in diameter were employed. Six-inch pots of air-dry soil were divided into groups which were kept dry for various periods, as indicated in Table 4, and then moistened and planted with tomato seed. In the check pots, however, the soil was moistened immediately after the galls were introduced into the air-dry soil. All groups were in replicates of five. In experiment 1, positive infection was obtained in each of the check pots. The second group (galls in air-dry soil for two weeks) gave

TABLE 5—*Effect of various soil moistures on nematode galls from tomato, attached and detached galls—Continued*

## SERIES E, 100 PER CENT MOISTURE CONTENT

Period galls were in soil (weeks)	Attached galls	Detached galls
1.....	Soft, gall intact.....	Very soft, central cylinder exposed due to sloughing of tissues surrounding it.
2.....	Galls whole, very mushy.....	Galls whole but all decayed under epidermis, central cylinder hard.
3.....	Epidermis whole and tough, fleshy tissues all gone, hard central cylinder	Epidermis whole and soft, hard central cylinder
4.....	Epidermis whole, tissues missing, central cylinder soft	Epidermis whole, tissues missing, central cylinder tough and hard but not brittle
5.....	Epidermis whole and tough, tissues missing to pliable central cylinder	Epidermis whole and tough, tissues missing to brittle central cylinder

At every moisture content, the attached galls were less susceptible than the detached to the natural forces of decomposition. As the moisture content of the soil increased, the process of decay was hastened in both the attached and detached galls. There was no decay in the containers entirely devoid of soil moisture. The galls at 0 per cent moisture gradually shriveled, the detached galls lost practically all their moisture and became brittle. At the end of the first week the attached galls were turgid at all moisture contents except at 100 per cent. In this container the galls were still intact, but their softness was evidence that the cells were ready to collapse. The detached galls in the same container were at this time (after one week) so far gone that the central cylinder was exposed, with the surrounding tissue dropping away.

A comparison of the progress of decay in the soils of different moisture content indicates that decay in both attached and detached galls at 100 per cent was 1 week ahead of that in galls at 60 and 80 per cent and 2 weeks ahead of that in galls at 40 per cent. However, when the rates of decay of attached and detached galls are compared, it is evident that the difference in rate of decay decreases with increase in moisture content of the soil. At 100 per cent moisture content, the attached galls lagged about 1 week behind the detached in the process of decay; at 80 per cent, the lag was 2 weeks; at 40 and 60 per cent, 3 weeks; and at 0 per cent, 5 weeks or more.

## SUMMARY

This paper presents a study of the effect of environment on the activity of the root-knot nematode, *Heterodera radicumicola* (Greeff) Mull., of the tomato gall.

The nematode gall of the tomato plant is well suited as material for environmental investigations. It offers substantial protection to its nematode population because of the resistant root-covering tissue.

All stages in the life cycle of the nematode may be found in a decaying gall. Among these, there was found infrequently a female nematode, which contained a mass of eggs. It was cystlike, and corresponds to the brown cyst form of the sugar-beet nematode, *Heterodera schachtii* Schmidt. This particular cystlike form was not found in galls from cucumber or lettuce plants, and only on tomato plants that were growing in a relatively dry soil.

soil. The water-holding capacity of the soil as determined by the method of Hilgard (8, p 209) was 53 per cent of its air-dry weight.

Growing plants galled by nematodes were planted in each container. The soil in the containers was maintained at 70 per cent moisture until all plants were well established. This took about three weeks. The low-moisture containers were reduced to their 0 and 40 per cent values in about 5 days. All weights were maintained by frequent weighing.

When all pots were adjusted to the proper moisture percentage detached galls from other plants were placed in cages of window screening and deposited in each container with some of the soil to insure the same environment as the attached galls. The living plants were then cut at the surface of the ground without disturbing the root system. At intervals of a week one container from each series was removed and the attached and detached galls were examined. The results are recorded in Table 5.

TABLE 5.—*Effect of various soil moistures on nematode galls from tomato, attached and detached galls*

SERIES A, 0 PER CENT MOISTURE CONTENT		
Period galls were in soil (weeks)	Attached galls	Detached galls
1.....	Turgid, not shrunken, galls apparently perfect	Slightly shriveled, inclined to be brittle
2.....	Indications of shriveling, but on the whole turgid	Very shriveled and dry, not brittle
3.....	Smaller galls shriveled, larger galls hard and turgid	Very shriveled, brittle
4.....	Shriveled, soft, not brittle.....	Do
5.....	Shriveled, punky, not decayed, not brittle..	Do
SERIES B, 40 PER CENT MOISTURE CONTENT		
1.....	Turgid, unchanged.....	Softening, inclined to be spongy
2.....	Slightly shriveled, slight decay.....	Soft decay, epidermis easily sloughs
3.....	Soft, punky, dry and fibrous.....	Very fibrous, not much remaining
4.....	Very punky, reduced in size, epidermis easily sloughs	Nothing left but tough central cylinder
5.....	Galls whole, epidermis easily sloughs, slight decay	Only central cylinder remains, not hard
SERIES C, 60 PER CENT MOISTURE CONTENT		
1.....	Turgid, unchanged.....	Very soft, inclined to be fibrous
2.....	Slightly shriveled, slight decay.....	Nothing left but central cylinder which is hard
3.....	Punky, sloughs to a hard central cylinder.....	Soft central cylinder
4.....	Epidermis whole, tissues to central cylinder missing, central cylinder soft	Central cylinder hard
5.....	Epidermis whole, tissue beneath gone as far as hard central cylinder	Central cylinder nearly destroyed, very fibrous and soft
SERIES D, 80 PER CENT MOISTURE CONTENT		
1.....	Turgid, unchanged.....	Very soft, mushy
2.....	Galls whole, mushy.....	Nothing left but soft central cylinder
3.....	Soft, epidermis tough, hard central cylinder.....	Soft central cylinder
4.....	Epidermis soft, somewhat decayed, inner tissues missing, central cylinder soft	Do
5.....	Epidermis whole and fairly tough, tissues missing to central cylinder which was pliable	Central cylinder whole and brittle

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The optimum range of soil temperature for tomato, 25° to 30° C, is also the optimum range for nematode activity. A low soil-moisture content, 40 per cent, combined with high temperatures, 24° to 30°, increased the number of galls in proportion to the size of the root system. Plants grown in a soil saturated with water had very few galls, especially at the lower temperatures, 15° to 21°. Such plants have long slender roots.

An apparatus is described for observing the invasion of a root by a nematode. The suggestion is made that the size of a root may aid or prevent invasion by nematodes.

The nematode in a detached gall may survive for one month or longer in soils ranging in moisture content from 10 to 100 per cent. The organism did not survive this period in an air-dry soil.

Flooding a gall-infested soil for 28 days did not eradicate the nematode.

Galls in an air-dry soil did not protect nematode life as long as 14 days, but did protect it as long as 10 days.

A detached gall decays more rapidly than a gall attached to a root system in situ. The rate of decay of attached and detached galls increases with an increase of moisture in the soil. The rate of decay of an attached gall lags about three weeks behind that of a detached gall in a normally moist soil, that is, one containing approximately 60 per cent moisture.

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## THE RELATION OF MYCORRHIZAE TO CONIFER SEEDLINGS<sup>1</sup>

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### INTRODUCTION

Foresters are becoming more and more interested in the physiological relationships between forest trees and soil-inhabiting fungi. Certain soil fungi have long been known to be detrimental to tree seedlings as, for example, species of *Pythium* and *Fusarium* which cause damping off of succulent young seedlings. But the belief that all soil-inhabiting fungi are injurious to seedlings is no longer tenable. It is now believed that certain failures in forest nurseries and certain of the indifferent results sometimes encountered in planting out nursery stock are not due to the presence of a fungus but on the contrary should be attributed to the absence of some particular fungus.

Many pathologists believe that the association of certain fungi with the young root tips of trees and other plants to form mycorrhizae constitutes a definite case of parasitism on the part of the fungus. Other investigators are equally convinced that this association of two dissimilar organisms is distinctly beneficial to both participants. In any event, it is inconceivable that the formation of mycorrhizae should have no effect whatever on the health of the higher plants when all, or nearly all, of their rapidly growing root tips are invaded by fungous mycelium, with consequent swelling and forking of the roots. The occurrence of mycorrhizae on an astonishingly large number of plant species makes this question of the physiological rôle of mycorrhizae unusually important.

### HISTORICAL DATA

The presence of fungus hyphae in the root cells of plants was described by numerous investigators during the first half of the nineteenth century. There were many speculations concerning the origin of these mycelial threads but no evidence of value until Reese (26)<sup>2</sup> in 1880 noted the apparent connection between a spruce rootlet and a truffle fungus, *Elaphomyces granulatus*.

In 1885 Frank (3) coined the word "mycorrhiza," and in a series of papers beginning in April of that year developed the hypothesis that a beneficial relationship might exist between the higher plant and the

<sup>1</sup> Received for publication Aug. 12, 1931, issued April, 1932. This contribution represents a dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy from the University of Michigan. The results here presented were obtained at the University of Michigan during the years 1927 to 1930. The work was started at the suggestion of C. H. Kauffman, to whom the writer is indebted for many helpful suggestions and much stimulating criticism.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 315.



thereafter steadily declines to a minimum during the winter months.

An imposing number of fungous species are reported as being mycorrhiza formers. Approximately 50 species representing 16 genera are in this list, and practically all are Basidiomycetes, chiefly agarics.<sup>4</sup> The genera mentioned most frequently are *Amanita*, *Boletus*, *Cortinarius*, *Cantherellus*, *Inocybe*, *Russula*, and *Tricholoma*. Unfortunately, very few of those who have reported certain fungi as mycorrhiza formers have based their opinions on experimental evidence. The writer can suggest at least a dozen species not yet reported as mycorrhiza formers that careful field examination discloses to be closely associated with Norway spruce (*Picea excelsa*) and northern white pine (*Pinus strobus*). It is evident that many statements encountered in the literature must be accepted with reservation until verified by laboratory experiments.

Two methods have been used to determine whether a certain fungus is a mycorrhiza former. By one method strands of mycelium from a fungous fruit body are followed through the soil to a mycorrhiza, by the other mycorrhizae are formed in pure culture by inoculating the roots of seedlings with fungous mycelium.

The chief objection to the first method is the great difficulty in following the strands of mycelium through the soil without breaking the filaments. Moreover, there is no assurance whatever that the mycelium followed to the root is the mycelium which is responsible for the formation of the mycorrhiza. As will be brought out later, even the presence of a fruit body directly on a root and apparently in the most intimate contact with it does not necessarily signify that the fungus forms mycorrhizae.

In the second method the mycelium designed for inoculation purposes is obtained either from cultures of known fungi or from cultures originating with a mycorrhiza. Inoculations are made on the roots of seedlings that presumably are sterile, or on the roots of seedlings germinating from disinfected seeds sown within a flask or other closed vessel. A few of the investigators—Fuchs (4), for example—who obtained mycelium from the fruit bodies, did so by germination of the spores, but usually this mycelium has been obtained directly from the fruit body by culturing tissue taken from the interior of the fruit body. To obtain a pure culture of mycelium from a mycorrhiza is a difficult task and can not always be accomplished. Melin has developed a method for culturing mycorrhizae which he asserts is successful in sterilizing the external parts of the root and thus excluding those species (e g., of *Penicillium*, *Fusarium*, and *Mucor*) which would be likely to outgrow the fungus causing the mycorrhiza. Fresh mycorrhizae are washed several times in sterile distilled water and then treated for 15 to 30 seconds with a 0.1 per cent solution of mercuric chloride, after which they are again washed in sterile distilled water and placed on the culture medium. Masui also has used this method with success. Moller (22) extracted mycelium from a spruce mycorrhiza and reinfected spruce rootlets with this mycelium. He inoculated with nonseptate mycelium which he thought might be a *Mucor*, but the mycelium which he later found inside the roots was septate. Moller's method of obtaining sterile rootlets for inoculation purposes

<sup>4</sup> According to Melin, certain incompletely identified species of Rhizoctonia may form "false" mycorrhizae. A few species of Ascomycetes have been reported as being mycorrhiza formers.

fungus It is now generally considered that knowledge of mycorrhizae begins with the publication of Frank's researches Frank, however, was not the first to advance the concept of a beneficial relationship between fungi and the roots of higher plants, for Pfeffer (24) in 1877 ascribed to the orchid fungi a physiological rôle analogous to that of root hairs, and Kamienski (9) in 1882 published a memoir on *Monotropa* in which he fully recognized the existence of a reciprocal relationship between flowering plants and fungi These works have been overshadowed considerably by Frank's publications

The interest in mycorrhizae that was aroused by Frank's papers has persisted and increased More than 300 persons have written a total of approximately 600 papers dealing directly or indirectly with this subject

It is now generally accepted that root infection of the mycorrhizal type is a widespread phenomenon among vascular plants Mycorrhizae have been found among the Pteridophytes on ferns and on *Equisetum*, they have been found on the roots of orchids, violets, heaths, and a host of other small plants, also, on the roots of a great many species of forest trees, both hardwoods and conifers, and of fruit trees

Several types of mycorrhizae have been pictured and described The three types usually mentioned are ectotrophic, endotrophic, and ectendotrophic The terms "ectotrophic" and "endotrophic" were first used by Frank, Melin (17, 18, 19, 21) originated the term "ectendotrophic" Ectotrophic mycorrhizae are common on forest trees, especially conifers They are characterized by a fungous mantle around the root tip and the presence of mycelium between the cells of the root In endotrophic mycorrhizae the hyphae are found inside the root cells and the hyphal strands pass through the cell walls from one cell to another, this type of mycorrhiza is found most often on herbaceous plants and hardwood trees The characteristics of the endotrophic and ectotrophic mycorrhizae are combined in the ectendotrophic form McDougall's (14) term "heterotrophic" is perhaps comparable to ectendotrophic Rayner (25) believes that the type of mycorrhiza formed depends upon the degree of infection of the root by the fungus and intimates that a given fungus might form ectotrophic mycorrhizae on one plant and endotrophic mycorrhizae on another Melin considers that the different types of mycorrhizae (ectotrophic, endotrophic, and ectendotrophic) represent phases in development He believes that the ectotrophic condition is the final stage in a gradual "squeezing out" process due to enzyme activity in the root cells The endotrophic mycorrhiza with its intracellular mycelium may, therefore, be transitional to the ectendotrophic type and this may be followed by the true ectotrophic type Melin also states that the type finally developed probably depends on the "virulence" of the fungus According to Melin, these intracellular filaments eventually disappear, and he assumes that they are digested and utilized by the root cells Masui (15, 16) describes this digestive process as consisting solely of a degeneration of the filaments due to the gradual granulation of the cell membrane of the hyphae until at last it refuses to take the stain and hence no longer can be seen

The seasonal occurrence of mycorrhizae has been studied by both McDougall and Masui These investigators found that mycorrhiza formation reaches a maximum in late summer or early fall and

reserve supply of nutrient solution is kept in the other flask. The object in using flasks is to make certain that the cultures remain absolutely sterile. Meln candidly admits, however, that in spite of all precautions at least 25 per cent of his flask cultures were contaminated by the end of the third growing season.

Nearly every experimenter has remarked upon the difficulty of obtaining a culture medium that is suitable for both seedling and fungus. Sand has been used chiefly because it is easy to sterilize and is inert. The latter characteristic is important when the effect of various nutrient substances on plant and fungus development is being tested. Although sand is suitable for the seedlings, some difficulty is usually experienced in getting the fungi to grow through it unless it is in very coarse particles. Humus and forest soils containing humus have been used, but these media are difficult to sterilize without seriously changing their chemical composition; often substances toxic to both seedlings and fungi are produced when humus is steam sterilized; chemical sterilization is considered impractical. Various attempts have been made to increase the porosity of sand used as a culture medium. Among the substances which have been mixed with it to make it more porous are glass beads and moss. Meln placed sand in flasks and sterilized these for 25 minutes in a steam sterilizer on each of three days. Fuchs also used sand in flasks and autoclaved these for 2 hours at  $150^{\circ}\text{C}$ , he then mixed the treated sand with the nutrient solution and again sterilized the filled flasks for one-half hour. Masui used sand, and also a mixture of sand, humus, and sphagnum moss. These media were autoclaved in flasks for 30 minutes at  $150^{\circ}\text{C}$ .

So far as is known, Meln's 3-year experiments are the only ones lasting more than one year. The shortest experiments probably are some of those performed by Fuchs, these lasted but eight days. Masui's experiments were ended two or three months after the inoculations. Von Tubeuf (31) made experiments continuing for one year.

The effect of the association of a root and a fungus is one phase of this subject which has had a great deal of attention and about which there is yet very little definite information. Since Frank first raised the question as to the probable value of the fungus to the higher plant, our knowledge of this phase has gone very little beyond the theoretical stage. Nearly every conceivable hypothesis has been advanced as to the rôle of mycorrhizae in plant nutrition. The investigators who hold that the formation of mycorrhizae is injurious to the host plants include Sarauw (29), Moller, (22), Fuchs (4), Masui (15, 16), and McDougall (14). Frank (3), Stahl (30), Von Tubeuf (31), Rexhausen (28), and a number of others think the formation of mycorrhizae is beneficial or even necessary to the best development of the associated higher plant.

For about 30 years there was little compromise between these diametrically opposed ideas. But in 1909, Bernard (2) suggested that there might be a "balance of benefit" between the associated organisms, and that under certain conditions the association was beneficial to both participants, whereas under other conditions evidence of parasitism would appear. Since 1917 Meln has further developed the "balance of benefit" idea and has attempted to show that whether or not a mutual benefit is derived from the mycorrhizae

was to make a column of three pots of sterile sand and start the seedlings in the highest pot, when the roots had grown downward through two pots and had entered the third, they were inoculated by smearing mycelium on them

Peklo (23) obtained a fungous culture from beech mycorrhizae by making a decoction of the mycorrhizae. Sections of beech roots which had been rinsed in water were dropped into this decoction and on these bits of roots he obtained a fungous mantle composed of several species of *Penicillium*. Peklo's results are of questionable value because he used seedlings which were grown for two years in unsterilized humus and may have been infected. Furthermore, Peklo and some of the other investigators failed to take into account the possibility that several species of fungi might be associated with the roots and none of them be able to form mycorrhizae. A few years later, in 1911, Fuchs found this to be true, and he declares that the fungous mantle sometimes developed by certain species of fungi is no sure evidence of mycorrhiza formation because the hyphae do not penetrate even the outer cells of the root, and the mantle is easily washed off. He cites *Penicillium* as an example of a fungus that forms a mantle which does not penetrate the root and hence is not mycorrhizal. When mycelium is extracted from a mycorrhiza there is no assurance that only one fungous species has been obtained, and unless the cultivated mycelium can be made to form a fruit body there is no way to know what fungus is being cultured. Finally, there is the possibility that the fungus really responsible for the formation of the mycorrhiza can not be extracted from the mycorrhiza, certain species (e. g., of *Amanita* and *Cortinarius*), which are strongly suspected of being mycorrhiza formers, grow very slowly or not at all in artificial culture.

Most of the recent investigators have begun their experiments with the seed, either raising seedlings under sterile conditions and transplanting these at the time of inoculation, or raising one or a few seedlings in a flask or test tube and inserting the mycelium into these vessels. The latter method was used by Meln and Fuchs. Masui has used both methods, sometimes inoculating the seedlings when transplanted and at other times several months after transplanting.

Some investigators disinfected the seeds before planting, but others have not taken this precaution. Seed disinfection methods have varied, but solutions of mercuric chloride appear to meet with most favor. Masui tried several methods and settled on one involving the washing of green cones (containing germinable seeds) in 50 per cent alcohol, then in a 0.1 per cent solution of mercuric chloride. The seeds were removed while the cones were in the mercuric chloride solution and were then washed in sterile distilled water. Fuchs' method was much more elaborate. The seeds were shaken in 50 per cent alcohol, then in concentrated sulphuric acid for 5 to 10 minutes. They were then washed in a suspension of calcium carbonate to neutralize the acid, were rinsed in water, immersed for five minutes in 1.0 per cent mercuric chloride solution, and again rinsed in water.

In the first experiments with mycorrhizae, Frank used earthen flowerpots and Moller also used pots. Most of the latest experimentation have been done in Erlenmeyer flasks. Meln has used a double flask made of one Erlenmeyer flask and one Florence flask connected by a glass tube. The culture is grown in the Erlenmeyer flask and a

experimental evidence does not always indicate a beneficial effect, it at least shows no parasitism on the part of the fungus. A full discussion of this phase of the subject is given in later paragraphs.

#### FIELD EXAMINATIONS

In order that laboratory experimentation might be tempered by a knowledge of mycorrhizal conditions as they obtain in nature, a systematic study was made in several forest plantations. These were located on the Saginaw Forest of the School of Forestry and Conservation, University of Michigan. After a preliminary examination in 1927 and 1928, when every conifer plantation was visited regularly several times each week over a period of about three months in the fall and again in the spring, it was decided to restrict the study to the plantations of Norway spruce and northern white pine.

Mycorrhizae were moderately plentiful in the white pine plantation and were truly abundant in the spruce plantation. The abundance of spruce rootlets in the top 6 inches of soil and the moist conditions prevailing in this stand undoubtedly provide a more favorable environment for the formation of mycorrhizae than the rather dry soil of the pine plantation. An estimate of the total amount of mycorrhizal infestation in each plantation was made periodically by digging up a number of small roots about 3 feet long in the top 6 inches of soil. At least 98 per cent of the rootlets on the better quality sites of the spruce plantation, and probably 75 per cent of the rootlets in the white pine plantation, were estimated to bear mycorrhizae. In neither stand were mycorrhizae found very far below the surface, only to a depth of perhaps 6 inches in the pine and to about 8 inches in the spruce. No mycorrhizae were found in that part of the spruce stand where raw humus is absent and the soil bakes hard and dry.

The pH values for soil and humus were determined in these two plantations in the fall of 1928, the spring of 1929, and again in the fall of 1929. Samples from different parts of the stands were tested promptly with the quinhydrone electrode. In Table 1 are given the average pH values for soil and humus in these two stands.

TABLE 1 —Average pH values for soil and raw humus from the northern white pine and Norway spruce plantations on the Saginaw Forest

Soil	Northern white pine		Norway spruce	
	pH value	Sam- ples	pH value	Sam- ples
		Number		Number
Raw humus.....	6.4 ± 0.03	12	6.3 ± 0.19	15
Soil at a depth of 4 inches.....	6.0 ± 0.03	18	5.3 ± 0.02	19

Hesselman (7, 8) and Melin (20) obtained pH values of about 4 in the humus layers of conifer forests in northern and central Europe and Glømme (5) reports essentially the same values for conifer woods in Norway. According to Melin, Brenner found that soils in the conifer forests of Finland had pH values ranging from 3.5 to 4.8. In Sweden, Melin states, the best mycorrhizae and the most prolific occurrence of mycorrhizae are in forests where the raw humus has a pH value of

association depends very largely on the activity of the fungus. He points out that the various species of fungi which form mycorrhizae are not equal in their ability to form this union, but that some species are exceptionally vigorous while others are much less so. Furthermore, the external conditions which influence the health of the higher plant also have a part in determining in which direction the reacting organisms will proceed. Melin thinks that if a sickly plant effects an association with a particularly vigorous fungus, the plant may be injured and derive no benefit from the association, in a word, the fungus is parasitic upon the other plant. In short, the modern point of view is not that the formation of mycorrhizae must be either helpful or harmful to the participants of the association but that the effect of this association varies from absolute parasitism to complete symbiosis, depending upon the vitality of the fungus and the health of the higher plant.

The contradictory results obtained in investigations of the physiological significance of mycorrhizae probably are due to the methods used. In general, four methods have been employed to determine the effect on the plant of the fungus-root association. (1) Conclusions have been drawn from field observations. This admittedly is an inaccurate method, because allowance can not be made for all the many factors which simultaneously are influencing the development of the plant. (2) Conclusions have been based on the anatomical structure of mycorrhizae, but mycorrhizae are difficult to section and stain and even in a perfectly prepared slide it is easy to overlook important details. Many of the conclusions on the physiological rôle of mycorrhizae deduced from microscopic examinations should be verified by experiments with living plants. (3) Microchemical analyses have been made of infected and (supposedly) uninfected roots. Weyland (33) used these methods in studying the distribution of inorganic nutrient substances in root cells, and Masui has recently contributed an extensive paper on the subject (16). (4) There is the experimental method in which plant roots are brought into contact with the mycelium of a fungus suspected of forming mycorrhizae. By varying the conditions under which the plants are grown (as, for example, by supplying organic nitrogenous compounds to some plants and inorganic nitrogenous compounds to others), and by measurement of seedling height and needle length, observation of foliage color, and the like, direct evidence is obtained on the effect of mycorrhiza formation. In this field Melin is the outstanding investigator.

The investigators who have used the microchemical methods agree that the mycorrhizal fungi are parasitic upon the associated higher plants, and that there is no evidence of a symbiotic relationship. Masui, for example, found that the fungi remove from the root all of the amino acids, most of the carbohydrates, tannins, and nitrates, and some of the phosphorous, potassium, and ammonium. He found that young fruit bodies of the suspected fungi contained large amounts of these substances. Inasmuch as most of the important food substances were removed from the seedling by the fungus and no return of nutritive material was made, Masui was forced to conclude that the mycorrhizal fungus must be considered a parasite upon the seedling.

The evidence obtained by the experimental method is almost the exact opposite of that obtained by microchemical analyses. If the

## Ectotrophic mycorrhizae

## On Norway spruce—

*Amanita muscaria* Fr  
*Lycoperdon pulcherrimum* B and C  
*Inocybe eutheloides* Pk  
*Clitocybe rivulosa* Fr var *angustifolia* Kauff  
*Clitocybe diatreta* Fr  
*Calvatia saccata* Vahl  
*Boletus piperatus* Bull

## On white pine—

*Cortinarius* sp.<sup>5</sup>  
*Calvatia saccata* Vahl  
*Lycoperdon gemmatum* Batsch  
*Collybia butyracea* Fr  
*Inocybe eutheloides* Pk  
*Clitocybe rivulosa* Fr var *angustifolia* Kauff  
*Clitocybe diatreta* Fr.

## Ectendotrophic mycorrhizae

## On Norway spruce—

*Cortinarius argentatus* Fr  
*Cortinarius cinnamomeus* Fr  
*Tricholoma personatum* Fr  
*Lepiota naucina* Fr (or ectotrophic only?)  
*Lycoperdon gemmatum* Batsch (or ectotrophic only?)

## On white pine—

*Lycoperdon gemmatum* Batsch (or ectotrophic only?)

Of these species, *Amanita muscaria* has been reported by Melin to form mycorrhizae on *Betula*, *Larix*, *Pinus sylvestris*, and *Picea excelsa*; *Cortinarius cinnamomeus* has been reported by Masui to form mycorrhizae on *Pinus densiflora* and *Populus tremula*; the rest, so far as is known, are mentioned for the first time in this connection.

It has been the common practice of investigators to consider the attachment of fruit bodies to tree roots as definite proof of the ability of these fungi to form mycorrhizae. It is realized that circumstantial evidence of attachment is often very strong, but the apparent attachment of fruit bodies to tree roots can not be taken as proof that the fungus forming the fruit body likewise formed the mycorrhizae. The writer has made a microscopic examination of 28 fruit bodies of *Lycoperdon gemmatum*, *Amanita muscaria*, *Calvatia saccata*, *Inocybe eutheloides*, *Lycoperdon pulcherrimum*, and *Cortinarius* sp., each with tree rootlets attached. In spite of careful sectioning, staining, and examination, in none of over 100 slides could the mycelium of the fruit body be followed to the interior of the root (13).

One hundred and eight individual collections of mycorrhizae from the white pine and Norway spruce plantations were embedded in paraffin, sectioned, and stained. There was sectioned also one collection from each of the following species: Austrian pine (*Pinus nigra* Arnold), Scotch pine (*P. sylvestris* L.), ponderosa pine (*P. ponderosa* Laws), Douglas fir (*Pseudotsuga taxifolia* Britt), and eastern hemlock (*Tsuga canadensis* (L.) Carr).

The mycorrhizae found in the spruce plantation were simple or coralloid. The simple form is doubtless an early stage of the coralloid form; the root is unbranched and the mantle is very thin. The coralloid form (the "Gabelmykorrhiza" of Melin) is characterized by a corallike branching of the rootlets and the presence of well-developed hyphal mantles.

<sup>5</sup> An undescribed species which Doctor Kauffman has had under observation for several years.

about 4. He also states that usually there is an abundance of mycorrhizae in spruce woods having pH values of about 5 for the soil, but that where the pH value is 6 or 7 the development of mycorrhizae is very weak. Melin's experiments lead him to believe that vigorous formation of mycorrhizae will not take place on neutral or on alkaline soils or in extremely acid soils (e. g., with a pH of about 3.5 or less). Lohman (12) has made a large number of collections of mycorrhizae from a wide variety of plants in Iowa and has determined the pH values for the soil surrounding each collection. These values ranged from 4.9 to 8.2 but were 6.5 or over in the case of more than 70 per cent of his collections. He concludes that mycorrhizae occur in acid, neutral, and alkaline soils but probably develop better and are more common in soils having pH values below 6.5.

The "glass plate" method of studying the seasonal development of mycorrhizae—first mentioned by McDougall—was tried in the spruce stand but was not entirely satisfactory for this purpose. Judging, however, from a large number of individual collections, the maximum development of mycorrhizae in these plantations occurs from early September to late November, during three successive years the best collections were had in October. The development of mycorrhizae apparently depends on abundant rainfall and warm weather, after a hot, dry summer a considerable fall of rain is needed to start a vigorous growth of the fungous mycelium. Excellent mycorrhizae were obtained two weeks before the appearance of fruit bodies in these areas. New mycorrhizae probably do not develop after the advent of cold weather and frozen soil, but those already formed may remain alive for several months, a number were dug from frozen soil in December, January, and February. By spring nearly all those formed during the previous year have turned brown, are more or less shriveled, and appear to be dead. Only a few mycorrhizae are formed during the spring and summer months.

In the Norway spruce stand were found seven 1-year-old spruce seedlings which apparently had originated naturally from seeds cast by the trees of this stand. No mycorrhizae were found on the roots of these seedlings.

In both the Norway spruce and northern white pine stands a great many attempts were made to verify the apparent connection between fungous fruit bodies and mycorrhizal roots. Many fruit bodies were found in actual contact with roots; others were directly over large mycorrhizal clusters and separated from the roots by only a centimeter or so. This apparently intimate association is no positive assurance that the mycelium which formed the fruit body also is responsible for the near-by mycorrhizae, but these associations do furnish a clue as to which fungi might well be tested experimentally to determine their mycorrhiza-forming ability. In the following list are given the names of species<sup>4</sup> which careful and repeated examination disclosed to be nearly always associated with tree roots in these two stands. The list also indicates the fungi suspected of forming mycorrhizae of the different types found in the microscopic examinations of specimens from the two plantations. These species represent only a part of the large number of fungous species collected in the two areas.

<sup>4</sup> The identification of these fungi, without regard to the mycorrhizal types they may form, has been checked by C. H. Kauffman.



Longitudinal sections through mycorrhizae on Norway spruce, suspected of being formed by *Cortinarius argentatus*, showing mycelium within cells of the root. A, magnified 700 times, B, 260 times

The mycorrhizae of spruce, which presumably are formed by *Amanita muscaria*, are markedly coralloid in form. At maturity they are invested with a mantle of pure white mycelium which turns brown with age and eventually sloughs off as the mycorrhizae die and begin to shrivel. The mycorrhizae are about medium size, from 3 to 5 mm long. Microtomic sections show the hyphal mantle to be comparatively thick, from  $15\mu$  to  $30\mu$ , and composed of a tangle of hyphal filaments which lie closely appressed to the rootlet. The mycelium penetrates between the cortical cells of the root as far, usually, as the central cylinder.

The mycorrhizae of Norway spruce, which probably are formed by *Cortinarius argentatus*, are coralloid and have a yellowish-white mantle. Numerous short hyphal projections give these mycorrhizae a hairy appearance. The mycelium of the mantle soon turns brown, then blackish, and finally disappears as the mycorrhizae die. Microscopically, these mycorrhizae are noteworthy because of the presence of mycelium within the cells as well as between the cells (Pl. 1, A and B). Until recently it was not believed that ectotrophic mycorrhizae might contain intracellular hyphae, but as Melin has pointed out, this frequently occurs, the proper staining is necessary to make the filaments visible. In this particular mycorrhiza, thought to be formed by *C. argentatus*, no evidence of digestion of the hyphae could be found.

The mycorrhizae of Norway spruce which *Lycoperdon gemmatum* is suspected of forming are coralloid, but less so than those apparently produced by *Amanita muscaria*. They are about 5 mm long and have a cream-colored mantle with numerous projecting hyphal filaments. The mycelium is intercellular, but numerous short, stubby, haustoriallike branches of hyphae apparently penetrate the cell walls. It could not be determined whether actual penetration of the walls took place or whether the walls were distended inward without penetration. The hyphae were everywhere between the cell walls of the cortex but did not enter the central cylinder of the root.

*Tricholoma personatum* is suspected of forming pale-yellow mycorrhizae on Norway spruce. These mycorrhizae are distinctly coralloid and are characteristically bulbous at the tips. The swollen tip is sometimes twice the diameter of the rest of the mycorrhiza. The mycorrhizae are rather long, from 5 to 7 mm, and the mantle is smooth, from  $10\mu$  to  $20\mu$  in thickness, and composed of closely appressed filaments. The mycelium is both intracellular and intercellular, thus placing this mycorrhiza in the ectendotrophic class.

The mycorrhizae of spruce thought to be formed by *Chitocybe rivulosa* var. *angustifolia* are sparsely coralloid and were the least branched of all the mycorrhizae studied. When fresh, the mycelium of the mantle is grayish white and slightly fluffy, the mantle turns brown with age and appears to loosen and slip from the root as the mycorrhiza dies. The mantle is from  $8\mu$  to  $15\mu$  in thickness. These mycorrhizae are short, from 2 to 4 mm long, and the mycelium is exclusively intercellular, penetrating the root as far as the central cylinder.

Some of the mycorrhizae found in the northern white pine plantation were fully as coralloid as the *Amanita muscaria* (Norway spruce) mycorrhizae, but others found in this stand were almost tuberculate (the Knollenmycorrhiza of Melin). In no instance, however, were these tuberlike mycorrhizae joined together, as often occurs with the

Knollenmykorrhiza type Where the same (suspected) species of fungi were present as appeared in the spruce areas, the white pine mycorrhizae had much less conspicuous mantles, although microscopic examination showed that the mantles often were just as thick

The mycorrhizae of white pine supposedly formed by *Collybia butryacea* were rather short (about 3 mm) and were invested with a grayish mycelial mantle from which numerous short filaments protruded The mycelium penetrated between the cells of the cortex occasionally as far as the central cylinder, no intracellular hyphae were found The mycelium inside the root was distinctly darker than the mycelium composing the mantle

The mycorrhizae of white pine suspected of being formed by *Calvatia saccata* were tuberculate with occasional coralloid forms These mycorrhizae were 4 or 5 mm long and had a thick mantle of cream-colored mycelium which appeared to darken with age; the mantle was not found on old (apparently dead) mycorrhizae This mantle was from  $12\mu$  to  $20\mu$  in thickness and was composed of very closely interwoven filaments The mycelium was exclusively intercellular and penetrated the root as far as the central cylinder It was much darker inside the root than outside

#### FORMATION OF MYCORRHIZAE UNDER CONTROLLED CONDITIONS

##### GROWING SEEDLINGS IN THE LABORATORY

Seedlings of Norway spruce and white pine were grown in pots of sand supplied with nutrient solutions The seeds were first disinfected by covering them with a 0.25 per cent solution of mercuric chloride and shaking vigorously for a few moments to insure perfect contact of the solution with every seed After 45 minutes the solution was poured off and the seeds were rinsed in three changes of sterile distilled water The disinfected seeds were planted in quartz sand The sand and the new earthen pots containing it had been sterilized by autoclaving for  $3\frac{1}{2}$  hours at 15 pounds pressure ( $121^{\circ}\text{C}$ ) Northern white pine seeds, especially when fresh, were extremely erratic in germinating, but alternate wetting and drying greatly hastened their germination For the spruce the germination averaged about 65 per cent and for the white pine about 50 per cent The age of the seedlings has been computed from the time when germination began to show a marked decrease

The nutrient solution used was that developed by Reid (27)<sup>6</sup> When a solution without nitrogen was required, the nitrates of the Reid solution were omitted For solutions having nitrogen from an organic source, the nitrates were replaced by asparagine, glycine, uric acid, or peptone These solutions were applied to the seedlings every 10 days The plants were watered with distilled water.

Various experiments were made to determine approximately the requirements of the seedlings for light Direct sunlight was kept from the seedlings because of the considerable increase in temperature accompanying sunlight. Artificial light (supplied by 50 w electric lamps) was used to supplement the indirect sunlight reaching

<sup>6</sup> The chemical composition of the solution is as follows: Solution A— $\text{MgSO}_4$ , 2 per cent,  $\text{KH}_2\text{PO}_4$ , 2 per cent,  $\text{KNO}_3$ , 2 per cent. Solution B— $\text{CaCl}_2$ , 3 per cent,  $\text{CaSO}_4$ , 2 per cent,  $\text{Ca}(\text{NO}_3)_2$ , 4 per cent. Equal quantities of the solution are mixed and then diluted 20 times. A few drops of 1 per cent ferric citrate solution is added to the diluted solution.



*Calvatia saccata* Vahl  
*Cortinarius* sp  
*Lycoperdon gemmatum* Batsch  
*Amanita muscaria* Fr  
*Boletus piperatus* Bull  
*Cortinarius cinnamomeus* Fr  
*Collybia butryacea* Fr

The initial cultures and the subcultures in test tubes were made on a nutrient agar.<sup>8</sup> Tests were made to determine the effect of various nutrient substances on the growth rate of the fungi.<sup>9</sup> Among the media used in these experiments were the following: Sugar agars which contained only matose, glucose, or sucrose and agar and water, an agar containing the compounds included in the Reid nutrient solution for seedlings, humus agars containing a decoction made by boiling raw humus from the spruce plantation, asparagine agar, in which the nitrogenous compounds of the regular nutrient agar were replaced by asparagine, Leonian agar;<sup>10</sup> malt extract agar, and several "tannin" agars made by adding to the media mentioned a little concentrated solution (of tannin and probably other substances) extracted from cork by boiling it in water.

Sucrose appeared to be less adapted to the needs of these fungi than either maltose or glucose, but no appreciable growth of mycelium was obtained unless a sugar was included in the culture medium. Most of the fungi made an extensive growth of mycelium on agar containing only sugar, but satisfactory development over a long period was not obtained unless mineral salts also were available. Although these fungi thrive in humus soil, none of them lived on the agar containing humus decoction, it is possible that boiling the humus may have produced substances toxic to the fungi. The addition of a little concentrated cork extract (probably consisting chiefly of tannin) to the culture medium materially aided the mycelial development of all fungi. The regular nutrient agar (see footnote 8) and this agar with cork extract produced the most rapid development of mycelium.

The individual fungi varied greatly in rate of mycelial growth. *Clitocybe rivulosa* made the most rapid spread of mycelium; this fungus grew rapidly from the time that cultures were started. Other species—e. g., *Calvatia saccata*—spread very slowly for several days and then grew vigorously. *Boletus piperatus*, *Amanita muscaria*, and *Cortinarius* sp. made no appreciable growth on any of these media.

The mycelium developed most rapidly at temperatures approximating 22° C. At 15° all fungi grew very slowly, and mycelial spread likewise decreased very markedly when the cultures were kept at 30°. At 36° there was almost no increase in spread of the mycelium.

These results are for fungi in artificial culture and may not represent the actual development of the fungi when growing in their native habitats. *Amanita muscaria*, for example, normally may be a slow-growing species, but it undoubtedly grows much faster in nature than

<sup>8</sup> This agar had the following composition: Agar, 15 g, maltose, 5 g, peptone, 0.1 g, MgSO<sub>4</sub>, 0.5 g, Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 g, KH<sub>2</sub>PO<sub>4</sub>, 0.25 g, distilled water, 950 cm<sup>3</sup>.

<sup>9</sup> The cultures were kept at a uniform temperature of 22° C. and the mean radial growth of the mycelium was measured every day. For about half of the cultures an additional measurement was made by tracing the daily spread of the mycelium with wax pencil on the bottom of the Petri dish; these impressions subsequently were transferred to sheets of paper and the area of each "ring" obtained with a planimeter; this indicated the daily spread of mycelium in square inches. The results thus obtained agreed very closely with those obtained by measuring the mean radial growth.

<sup>10</sup> Leonian agar has the following composition: Agar, 15 g, malt extract, 6.25 g, maltose, 6.25 g, MgSO<sub>4</sub>, 62 g, KH<sub>2</sub>PO<sub>4</sub>, 1.25 g, distilled water, 1,000 cm<sup>3</sup>.

<sup>11</sup> g and cm<sup>3</sup> are the abbreviations recently adopted by the Government Printing Office for grams and cubic centimeters, respectively.

the seedlings. It was found by experiment that continuous light—at least, over a period of one month—was not harmful to the seedlings, on the contrary, continuous illumination stimulated the development of new foliage.

Experiments were made to learn at what air temperature and relative humidity the best development of the seedlings takes place and at what point the seedlings begin to suffer from the influence of these two factors. It was found that rapid growth occurred with temperatures approximating 18° to 22° C and a relative humidity above 35 per cent. The seedlings suffered when the temperature was above 24° and also when the relative humidity was below 20 per cent. The harmful effect of heat was offset by a high relative humidity, the maximum safe temperature being about 30° in a saturated atmosphere. It was observed also that a moist atmosphere encourages the development of new foliage and that dry air retards it. Because of this, the air temperature of the laboratory was kept as near 20° as possible, the lights were not used when the temperature unavoidably rose above 22°, and the air was kept moist by sprinkling water on the floor. Unless these precautions are taken it is likely that seedlings will die, and in some instances their death might erroneously be attributed to the action of a fungus if culture experiments are in progress.

In an experiment to determine the effect of the nitrogen source on the development of the seedlings, one set of seedlings was supplied with inorganic nitrogenous compounds, another set was furnished nitrogen in organic compounds (asparagine, peptone, glycine, and uric acid), and a third set was supplied with a nutrient solution containing no nitrogenous compounds. The seedlings with nitrates developed normally; at the end of the experiment (six months) they had comparatively short but many-branched roots, foliage of a dark-green color, and had increased about 1½ inches in height. The seedlings without nitrogen had a sickly appearance, their roots were long and sparsely branched, the foliage was yellow with many needles dropping, and they had grown scarcely half an inch in height. The seedlings supplied with nitrogen from organic compounds likewise exhibited signs of nitrogen starvation.

#### GROWING FUNGI IN PURE CULTURES

A number of fungi were cultured by placing fragments of clean tissue from the interior of fruit bodies on nutrient agar in Petri dishes. When sufficient mycelium had developed in these initial cultures, subcultures were made in other dishes and in test tubes. The best cultures were obtained from fresh, young fruit bodies collected during the period of maximum fruit-body production (in October), mycelium obtained from fruit bodies collected much earlier or later grew very slowly. The following fungi were cultured:<sup>7</sup>

*Clitocybe rivulosa* Fr. var. *angustifolia* Kauff.

*Clitocybe diatreta* Fr.

*Tricholoma personatum* Fr.

<sup>7</sup> There was no reason to expect variations in the cultures of any fungus due solely to the location from which the fruit body was obtained. As a matter of record, the inoculation experiments to be described were made with cultures of fruit bodies obtained from the white pine and Norway spruce plantations, as follows: *Lycoperdon gemmatum* Batsch, from spruce stand, Nov. 8, 1928; *Tricholoma personatum* Fr., from spruce stand, Oct. 17, 1928; *Clitocybe diatreta* Fr., from pine stand, Nov. 4, 1928; *Calvatia saccata* Vahl, from spruce stand, Nov. 8, 1928; *Clitocybe rivulosa* Fr. var. *angustifolia* Kauff., from pine stand, Oct. 4, 1928.

layer of granulated cork, (3) a layer of cork mixed with sand, or (4) a cork and sand mixture over a thin layer of agar bearing a vigorous growth of mycelium<sup>12</sup>. Disinfected seeds were planted in some of the flasks, sterile seedlings were transplanted to others.

The same species of fungi and seedlings were used as in the test-tube cultures to form mycorrhizae in these flasks. It was impossible, however, to continue the experiments for more than a few months because the humidity of the air within the flasks so promoted the aerial growth of the mycelium that the seedlings were smothered. Another difficulty was lack of drainage, seedlings and fungi died from excess moisture in the culture medium. A third difficulty was the rapid development of *Penicillium* and other contaminating organisms within the flasks—these pests did not appear in open cultures. Finally, it was impossible to maintain a large number of cultures.

An attempt was made to improve the moisture conditions within these flasks. For drainage, a short piece of glass tubing was fused into the side of the flask about half an inch above the bottom. To decrease the moisture content of the air within the flask an inverted U-shaped tube was inserted in the cotton stopper with one end of the tube inside the flask and the other end outside. These improvements overcame the excessively moist condition of the medium but did not materially reduce the atmospheric moisture within the flasks.

Simultaneous with these wholly inclosed cultures, another experiment was made using small (125 cm<sup>3</sup>) Erlenmyer flasks and leaving the foliage and most of the stem of the seedling protruding from the flask. One hundred and four of these flasks were used, filled to the top with sand or with the cork and sand mixture. Half of the flasks were treated with a nutrient solution containing nitrogen in inorganic compounds (nitrates), and the others were given a solution containing nitrogen in an organic compound (asparagine). By leaving the foliage of the seedlings outside the flasks the aerial growth of mycelium was prevented. The experiment lasted two months and was discontinued because of the excessive mortality of the seedlings. Mycorrhizae were formed on the seedlings (pine and spruce) by the four species of fungi previously mentioned, but it was found by experiment that the seedlings died not because of the formation of mycorrhizae but because of the moist condition of the media in the flasks. In most of the flasks the medium was either very dry or was soggy with moisture. The living seedlings were in the few flasks containing media that were moderately moist. These results again demonstrated that drainage is a most important factor, and seedlings later were successfully grown in flasks having drainage holes in their bottoms.

#### CULTURES IN POTS

The experiments thus far performed demonstrated that certain fungi form mycorrhizae on northern white pine and Norway spruce.

<sup>12</sup> An effort was made to find some material which would make the sand more porous. Among the substances were glass beads, glass wool, a mixture of agar and sand stirred together while hot and allowed to cool, granulated cork, and a mixture of cork and sand. The mixture of cork and sand was found to be very satisfactory. The cork was boiled in small quantities for 10 minutes in each of three changes of water to remove most of the tannin and other soluble substances. A test showed that 21 changes of water did not entirely remove the tannin but 3 changes of water removed a very large part of it. As the experiments with fungous cultures demonstrated, cork extract probably consisting mainly of tannin, does not inhibit the development of the fungi used in these experiments, on the contrary, it promotes the growth of the mycelium. After boiling, the cork was placed in flasks and autoclaved for one hour, then rinsed in two changes of sterile distilled water and dried at 105° C. for about four hours, or until dry. The mixture consisted of 1 part sand to 4 parts of granulated cork, by volume.

in these cultures. A year after these experiments were made they were repeated with approximately the same results.

#### FORMATION OF MYCORRHIZAE IN PURE CULTURES

Several investigators have produced mycorrhizae in pure cultures, though not always in abundance. A résumé of the methods used has already been given. In the experiments now to be described a variety of media and several types of containers were used with the object of finding the culture medium and type of container best suited to long-time experiments of this character. The media employed were agar, sand, sawdust, cork, and combinations of these. Test tubes, flasks, and earthen flowerpots were used as containers for the fungus-seedling cultures.

#### CULTURES IN TEST TUBES

Two types of cultures were made in test tubes. In one, disinfected seeds were placed on slanted nutrient agar and inocula added as the seeds germinated. In the other, a vigorous growth of mycelium first was obtained on slanted agar, then against this the roots of a sterile seedling were held in place either with sand or with sawdust.<sup>11</sup> When seeds were planted the tube was kept closed with a cotton stopper; when seedlings were used, the tube was filled to the top with the sand or sawdust and the top and most of the stem of the seedling protruded from the tube.

In these experiments with test tubes, mycorrhizae were formed on the roots of white pine and Norway spruce seedlings with *Tricholoma personatum*, *Clitocybe diatreta*, *C. rivulosa* var. *angustifolia*, and *Lycoperdon gemmatum*, none of which had been reported as a mycorrhiza former. The experiments gave no indication as to the physiological effect of mycorrhizae on the seedlings, for one set of seedlings (in sand) died while another set (in sawdust) lived when exposed to exactly the same species of fungi.

The experiments also revealed certain undesirable features in the use of test tubes. Raising seedlings from the seed within stoppered test tubes was not successful because aerial growth of the mycelium covered the foliage of the seedling. If sand is used as a restraining medium it must be used in larger quantity than can be contained in test tubes of the size employed (20 by 175 mm); unless this is done the sand will dry out and the seedlings will die. The indications are that sawdust is a better medium, physically, than sand for these experiments; it holds water better and, being more porous, permits a much better development of the mycelium than sand. In spite of these advantages, however, sawdust probably can not be used because of the difficulty of sterilizing it.

#### CULTURES IN FLASKS

The first flask cultures were continuations of the attempt to form mycorrhizae in wholly inclosed cultures. Erlenmyer flasks of 500 to 1,000 cm<sup>3</sup> capacity were used. One of the following culture media was used in each flask: (1) A layer of sand about 2 inches deep, (2) a

<sup>11</sup> Subsequent mention of "sterile" seedlings implies that the seedlings were grown from disinfected seeds under sterile conditions in sterilized sand and before transplanting to cultures were washed quickly in 95 per cent alcohol and rinsed in sterile distilled water.

Another experiment was started in which single seedlings were planted in 2½-inch and 3-inch pots instead of 5-inch pots. In this series only spruce seedlings and *Tricholoma personatum* were used. It was decided as a result of this experiment that the smaller pots for single plants are best, since, if a seedling dies during the course of the experiment, it may then be removed for examination without disturbing other seedlings. The results of this experiment are included with those obtained with the larger pots.

Artificial light was supplied to the seedlings for about eight hours each day. Distilled water was sprinkled over the pots daily in sufficient quantity to keep the sand layer moist. The small groove in the cork stopper allowed surplus water to drain away gradually but prevented water and nutrients from passing too rapidly through the pot. Nutrients were applied every 10 days by making a small hole in the sand layer and pouring the solution directly into the substratum through a funnel. In previous experiments it had been found that any of the nutrient solutions used in this experiment could be poured over pots of sand without danger of having such a common laboratory pest as *Penicillium* appear on the surface of the sand. These contaminations did appear, however, on the sides of the pots just above the sand layer where nutrient solutions had fallen in sprinkling them over the pots. By applying the solutions directly to the substratum the tops of the pots were kept clean throughout the course of the experiment.

The intrusion of *Penicillium* or other contaminating organisms was guarded against as much as possible, but there was no assurance that they would not reach the interior of these cultures, nor any reason to believe that these organisms would not grow in the cork and sand layer. At close of the experiment, however, not one instance of such contamination was found in these pots. Furthermore there is no known instance of *Penicillium* or like pests forming mycorrhizae. Fuchs made a number of attempts to form mycorrhizae with several species of *Penicillium* which he had extracted from roots bearing mycorrhizae but was unsuccessful. He reports that the mycelium of these fungi did not penetrate the roots and even if a hyphal mantle was formed it could be washed from the root.

To check Fuchs' results, 7 pots of seedlings were prepared as for the regular experimental work and were inoculated with cultures of fungi as follows: (1) 2 pots with *Penicillium* sp. from an agar plate exposed in the laboratory, (2) 1 pot with *Penicillium* sp. cultured from undisinfected Norway spruce seeds, (3) 1 pot with *Rhizopus* sp. cultured from undisinfected Norway spruce seeds, (4) 1 pot with *Rhizopus* sp. from an agar plate exposed in the laboratory, and (5) 2 pots with the only *Mucors* available, *M. lamprospora* and *M. spine-scens*. The pots were examined four months later and in no instance was there any evidence of mycorrhiza formation. Except for one pine seedling, the plants were all living in a healthy, vigorous condition. Microscopic examination of the roots of these seedlings, including the dead pine, disclosed no mycelium within the roots, and on only three seedlings was there an appreciable amount of mycelium on the exterior of the roots. As will be described shortly, mycorrhizae were formed in every pot inoculated with Basidiomycete fungi. It appears, therefore, that the ordinary contaminations likely to reach

The results gave several equally important clues as to the requirements for a method which could be used in extended experiments. Not only must this method be adapted to cultures lasting two or three years, but the method must be so devised that, the physical conditions existing in the cultures being kept as constant as possible, any observed change in the condition of the seedlings could be attributed to some other cause. A mixture of cork and sand had proved to be a suitable porous culture medium, drainage was found to be absolutely essential to avoid impossible growth conditions for the seedling, also, aerial growth of mycelium could apparently be prevented by permitting the foliage of the seedlings to grow in the open.

The use of earthen flowerpots as containers for the cultures was suggested very early by the results obtained in the experiments with test tubes and flasks. Preliminary tests with flowerpots were started as soon as difficulties were encountered in the use of flasks and the necessary foundation in experience was thus laid in time to take full advantage of the conclusions finally drawn from the flask experiments.

Sixty 5-inch pots were prepared in a uniform manner. A shallow, V-shaped groove (for drainage) was cut in a new cork along the entire length of one side and the cork inserted in the large drainage hole of a new clay flowerpot. The pot was autoclaved for  $3\frac{1}{2}$  hours at 15 pounds pressure. Sand was autoclaved in flasks for  $3\frac{1}{2}$  hours and then heated at  $105^{\circ}\text{C}$ . for about 6 hours, or until dry. New granulated cork was boiled in three changes of water, autoclaved, rinsed, and dried in the manner already described. Subsequent operations were performed as rapidly as possible and under sterile conditions. The sand and cork were mixed in the pot in the proportion of 1 part of sand to 4 parts of cork by volume. Sterile nutrient solution was added until the cork and sand mixture was thoroughly moistened. Mycelium from cultures made on agar containing only maltose was stirred into the cork and sand mixture. Seedlings raised under sterile conditions from disinfected seeds were washed in 95 per cent alcohol, rinsed in sterile distilled water, and transplanted to the pots. A layer of sterilized dry sand was poured over the top of the cork and sand mixture in each pot and moistened with distilled water.

Seven seedlings were placed in each pot. 3 spruce seedlings 3 weeks old, 3 spruce seedlings 5 months old, and 1 seedling of white pine 6 months old. The fungi used were *Tricholoma personatum*, *Clitocybe rivulosa*, and *Lycoperdon gemmatum*.

Nutrient solutions were applied in three forms: (1) With inorganic nitrogenous compounds (nitrates), (2) with organic nitrogenous compounds (asparagine, uric acid, peptone, or glycine), and (3) without nitrogenous compounds. Each nutrient solution contained 0.5 per cent maltose.

For the series with inorganic nitrogenous compounds there were 4 pots for each fungus species and a check of 4 pots without fungi, a total of 16 pots and 112 seedlings. For the asparagine series there were 4 pots for each fungus and a check of 4 pots without fungi, a total of 16 pots and 112 seedlings. For each of the uric acid, glycine, and peptone groups there were 2 pots for each fungus and a check of 2 pots without fungi, a total of 8 pots and 56 seedlings for each of the three groups. For the nitrogen-free series there was 1 pot for each fungus and a check of 1 pot, a total of 4 pots and 28 seedlings. In all, 420 seedlings were used.

The excellent seedling survival in these experiments with pots is noteworthy when compared with the results obtained in the experiments with flasks and test tubes. The survival with pots was over 98 per cent, and this includes the nitrogen-free cultures. Only two seedlings died in check pots, and these were in the peptone and the nitrogen-free series.

These experiments yielded no absolute proof that the presence of mycorrhizae is either detrimental or beneficial to the seedlings. Nor did the results support or refute the hypothesis that mycorrhizae aid seedlings to obtain nitrogen from complex organic compounds. The experiments did substantiate previous investigations which indicated that seedlings suffer from lack of nitrogen when this element is present only in complex organic compounds such as peptone.

#### DEVELOPMENT OF THE MYCORRHIZAL MANTLE

The development of the fungous mantle on seedling roots was studied in cultures made in glass-sided boxes. The most successful type of box was made of galvanized sheet iron and was 10½ inches tall, 8 inches wide, and 2 inches thick, with a sheet of plate glass in one of the larger faces. The glass surface was covered with black paper, except when an examination was being made. Difficulties in proper placement of seedlings were overcome as follows. A box was filled with sand and moistened thoroughly with nutrient solution, the box was then placed so that the glass side was uppermost and the glass was withdrawn from the box; the seedlings and inocula were placed as desired on the surface of the sand, and the glass plate carefully pushed back into the box. This box would be greatly improved if the sides were so hinged that the glass plate could be lifted directly from the sand and replaced without sliding.

In the tests made in these boxes mycelium on the roots grew very slowly unless in contact with the actively growing part of the root tip, when a pronounced increase in development promptly occurred. In most of the mycorrhizae the mycelium apparently reached the root at the tip, uniformly enveloped the tip, and then spread back over the root in a thin mantle at the rate of 2 or 3 mm a week. How rapidly the mantle increased in thickness is not known, nor has sufficient study been made to show how soon the mycelium begins to penetrate between the outermost cells of the root. In two months mycorrhizae were produced that were comparable to those of the regular pot cultures.

The location of the infected root tips was marked with wax pencil on the glass side, and in this way it was determined that the infected roots made no appreciable increase in length after the formation of the fungous mantle. The initial presence of the mycelium did not entirely stop the elongation of the root, but its rate of elongation suddenly decreased upon the formation of a rudimentary hyphal mantle.

Microscopic examination of older roots to which strands of hyphae apparently were attached disclosed no penetration of mycelium into the root cells. This indicates that mycorrhiza formation depends upon the presence of an actively growing root tip. Masui asserts that he found mycelium within older roots, but Melin was unable to verify this finding.

cultures in this particular laboratory may be disregarded in so far as mycorrhiza formation is concerned

The experiment with the large pots lasted four and one-half months. Mycorrhizae were formed on the roots of both pine and spruce seedlings in every inoculated pot. A few seedlings had less than 50 per cent of their rootlets infected, but on most of the seedlings all, or practically all, of the rootlets were infected with the fungous mycelium. The experiment again demonstrated that *Tricholoma personatum*, *Lycoperdon gemmatum*, and *Clitocybe rivulosa* var *angustifolia* form mycorrhizae on white pine and Norway spruce seedlings.

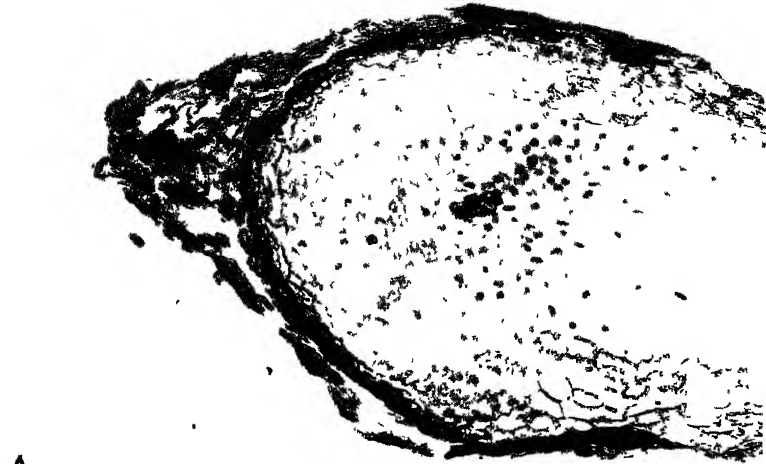
The experiment gave no indication that the nitrogen source (i. e., whether from organic or from inorganic compounds) has any marked influence on the rapidity of mycorrhiza formation or the abundance of mycorrhizae. Mycorrhizae were formed just as rapidly and in as large numbers in the cultures with peptone as a nitrogen source as in the cultures with nitrates. In the cultures where nitrogen was omitted from the nutrient solution, mycorrhizae were formed with no less rapidity but in somewhat less abundance than in the cultures with nitrogen.

In the series in which no nitrogen (except, perhaps, from the cork) was given to the cultures, the seedlings developed normally for nearly two months and then in both the inoculated and check pots the foliage began to fade and there was a slight decrease in the development of the needles. Two pine seedlings of the inoculated pots and one pine and one spruce seedling of the check pots were dead at the end of the experiment. Irrespective of the presence of mycorrhizae, these seedlings without nitrogen had a sickly appearance.

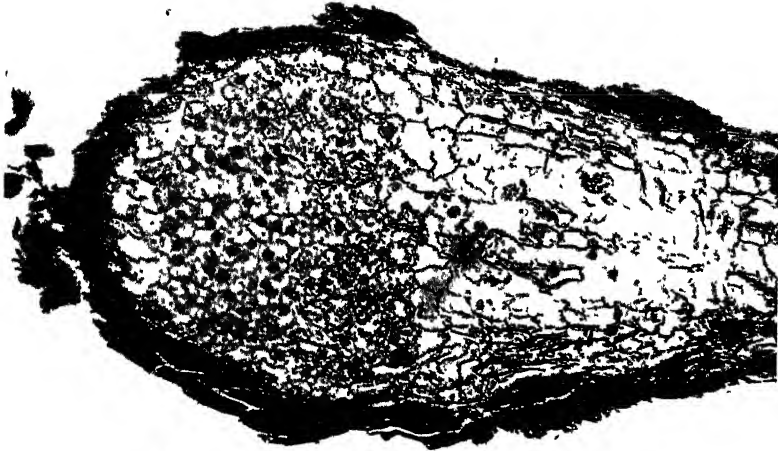
In the nitrate series the seedlings in both check and inoculated pots were in excellent condition at the end of the experiment. Their foliage was a fresh, green color and new needles were developing rapidly. Absolutely no difference could be seen between the infected seedlings and the uninfected seedlings. One pine seedling died at the age of six weeks in a pot inoculated with *Lycoperdon gemmatum*. The other seedlings, including those in the check pots, were alive at the end of the experiment.

In the asparagine, uric acid, and glycine series the results were almost the same as with the nitrate series. No difference could be detected between the appearance of the infected and the uninfected seedlings. The foliage of these seedlings was dark green and the needle development was vigorous. One pine seedling with asparagine and *Clitocybe rivulosa*, and one spruce seedling with uric acid and *Tricholoma personatum* were dead one month after the start of the experiment.

In the peptone series the results obtained were similar to those of the nitrogen-free series. The foliage was yellowish green and the needles appeared to be somewhat shorter than in the other series. Except for the color of the foliage, these differences were not pronounced. As nearly as could be determined, there was no appreciable difference in the condition of the infected and the uninfected seedlings. One pine of the check set and one young spruce with *Tricholoma personatum* were dead one month after the start of the experiment.



A



B

A and B, Longitudinal sections through mycorrhizae formed on Norway spruce in syntheses with *Tricholoma personatum* (peptone series)

The mycorrhizae produced in pure cultures were killed and fixed in weak chromo-acetic acid solution, after washing, dehydrating, and embedding in paraffin, these mycorrhizae were sectioned on a



FIGURE 1—Mycorrhizae on Norway spruce, formed in synthesis with *Clitocybe rivulosa* (asparagine series)

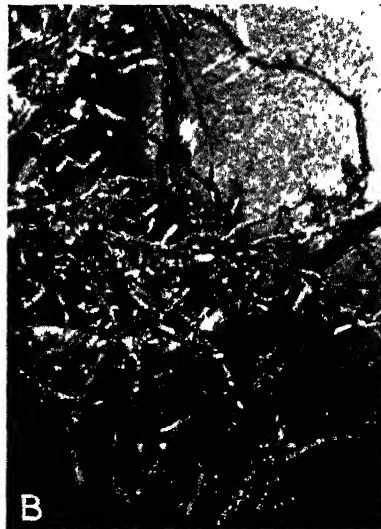


FIGURE 2—A, Mycorrhiza on Norway spruce, formed in synthesis with *Clitocybe rivulosa* (asparagine series), B, mycorrhizae on Norway spruce, formed in synthesis with *Clitocybe rivulosa* (nitrate series)

microtome and stained in Delafield's haematoxylin and safranin or with Pianeze III (32)<sup>13</sup>

<sup>13</sup> The stain has the following composition. Malachite green, 0.5 g, acid fuchsin, 0.1 g, naphthol yellow, 0.01 g; water, 150 cm<sup>3</sup>, and alcohol (95 per cent), 50 cm<sup>3</sup>

The mycorrhizae formed in the culture experiments closely resembled those collected in the field, except that the coralloid habit was less pronounced and the fungous mantles were somewhat thinner. The results so far obtained do not indicate any appreciable difference in the external appearance of the mycorrhizae which could be attributed to the nitrogen source.

The mycorrhizae formed on Norway spruce by *Clitocybe rivulosa* var. *angustifolia* were slightly coralloid, from 2 to 7 mm long, and had grayish white mantles with numerous short projecting strands of hyphae (Figs 1 and 2, A and B). The mantle was only  $4\mu$  to  $9\mu$  in thickness and was composed of loosely interwoven filaments about  $3\mu$  in diameter, frequently septate, and with numerous clamp connections. Within the root the mycelium was exclusively intercellular and penetrated the root in some mycorrhizae as far as the

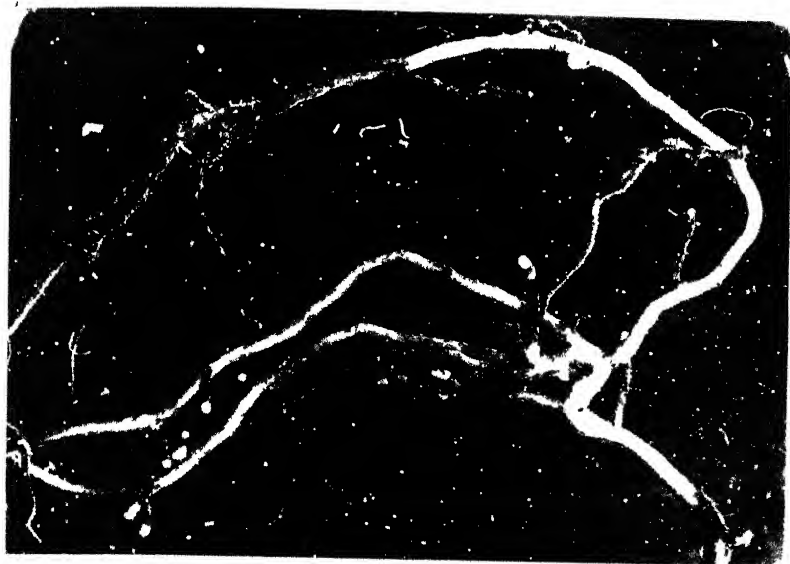


FIGURE 3—Mycorrhizae on Norway spruce, formed in synthesis with *Tricholoma personatum* (nitrate series)

central cylinder, this intercellular mycelium was darker than that of the mantle.

The mycorrhizae of Norway spruce and *Tricholoma personatum* (figs 3 and 4, and pl 2, A and B) were much more coralloid than those produced on spruce by *Clitocybe rivulosa* (figs 1 and 2). The mantles were yellowish white, with smooth outer surfaces, and were about  $10\mu$  or  $12\mu$  in thickness. The tightly interlaced hyphae of the mantles were about  $3\mu$  in diameter, had numerous clamp connections, and were frequently septate. Characteristic of these mycorrhizae were their distinctly bulbous tips, those collected in the field and suspected of being formed by this fungus also had bulbous tips. Except at the very tips of the roots the mycelium penetrated the root as far as the central cylinder and sometimes invaded that portion of the root. In practically all of the mycorrhizae examined, the mycelium was both intercellular and intracellular and was slightly darker inside



The mycorrhizae of Norway spruce and *Lycoperdon gemmatum* (fig. 5) were simple, but coralloid mycorrhizae occasionally were found. Their mantles were white, often with a pale-yellow tint, and the many short hyphal strands projecting from the mantles gave these mycorrhizae a hairy appearance. The mantles were approximately 6 mm long and from  $10\mu$  to  $16\mu$  thick. The mycelium was  $3.5\mu$  or  $4\mu$  in diameter, frequently septate but having only a few clamp connections and penetrating the root intercellularly almost to the central cylinder. In several instances an intracellular develop-

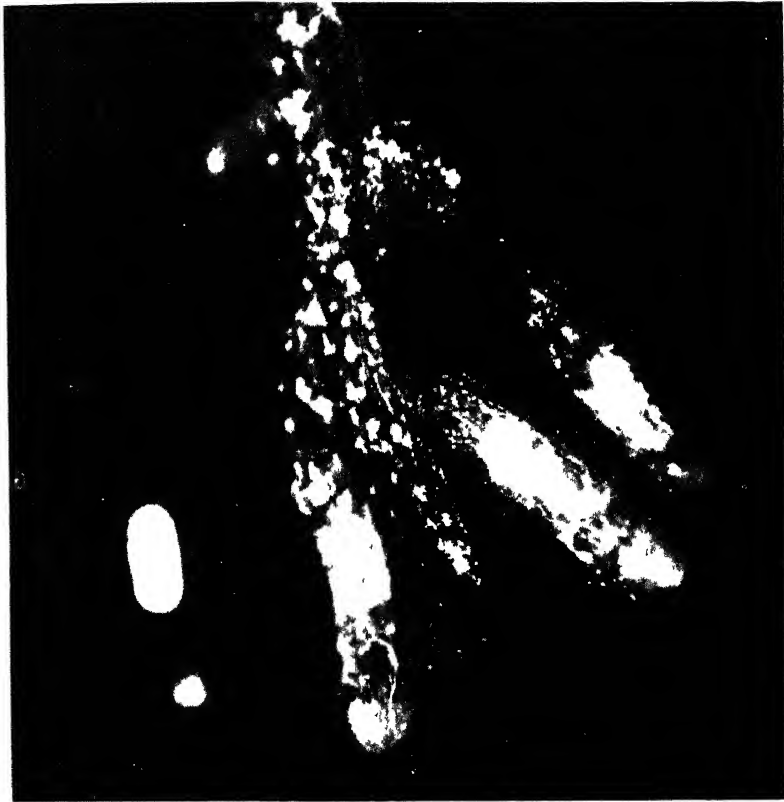


FIGURE 5—Mycorrhizae on Norway spruce, formed in synthesis with *Lycoperdon gemmatum* (nitrate series)

ment of the mycelium was suspected, but this could not be established definitely.

The mycorrhizae of Norway spruce and *Clitocybe dlatreta* were very similar to those formed on spruce with *C. rivulosa*, although the mantles were whiter. As with most of the mycorrhizae formed in culture the roots were but slightly coralloid. The mantles were composed of loosely interwoven hyphae about  $3\mu$  or  $3.5\mu$  in diameter, frequently septate, and with a great many clamp connections; numerous short filaments of hyphae projecting from the mantle

the root than in the mantle. No trace of the "digestion" of the intracellular hyphae could be found. The root cells in the cortical tissue contained minute granular bodies similar to those described by Masui, who expressed the opinion that these granules are com-



FIGURE 4—Mycorrhizae on Norway spruce, formed in synthesis with *Tricholoma personatum* (asparagine series)

posed of a tannic substance. Melin describes essentially the same condition in mycorrhizae of *Pinus sylvestris*, but thinks that the granular bodies are secreted by the fungus. Masui found them, however, in normal, uninfected roots.

height growth, size of leaf, or similar characters which usually vary widely, a large number of seedlings must be used in order to escape errors due to random sampling and to offset the inherent differences present in certain species of trees. Furthermore, there must be as much infection on the roots of these seedlings as occurs in nature; the formation of mycorrhizae on 5 or 10 per cent of the rootlets of a seedling does not simulate natural conditions. Moreover, when seedlings die it must be known without doubt whether death was caused by the formation of mycorrhizae or other factors.

Although there is but little definite proof, there are excellent indications as to the influence of mycorrhizae on seedling development. There are indications, for example, that the fungus prepares complex nitrogenous compounds for absorption by the plant. In an analysis of seedlings having peptone (an organic nitrogenous compound) as a source of nitrogen, Melin found that seedlings with mycorrhizae contained 8 per cent more nitrogen at the end of three years than uninfected seedlings in similar cultures. Just how this transfer of nitrogen is effected has not yet been determined. According to Hesselman (?), the detritus in a conifer forest (that is, in the "raw humus" type) decomposes very slowly, with the liberation of ammonia from the organic compounds. Very little is known of the chemical composition of humus, and it is difficult to discuss a reaction of this sort without accurate knowledge of the organic compounds present in humus. At all events, it is generally conceded that very little ammonia is produced in raw humus and that most of the ammonia which is produced is captured by the various microorganisms present. Melin states that in raw humus soils the nitrogen content is low in proportion to the carbon and for this reason the microorganisms can compete with the higher plants for nitrogen. He considers that mycorrhizae act as absorbing organs for the tree and thus enable the tree to obtain nitrogen in spite of the competition by the microorganisms. Frank (3), Hesselman (8), and Von Tubeuf (31) also assert that mycorrhizae aid the tree in absorbing organic nitrogen.

The experiments described in this paper suggest that seedlings without mycorrhizae have difficulty in obtaining nitrogen when it is present only in some complex organic compound. These experiments, however, do not prove that the presence of mycorrhizae enables seedlings to obtain nitrogen from such compounds. Cultures lasting for two or three years may modify this statement.

Melin, Stahl, and Rexhausen reported that inorganic compounds were absorbed equally well by infected and uninfected roots. Stahl and several other investigators have emphasized the importance of the fungous sheath in facilitating the passage of water into the root. As a matter of fact, Stahl considered that the formation of mycorrhizae represented an ecological adaptation to soil conditions.

Melin has shown that neither seedlings without mycorrhizae nor those with mycorrhizae can assimilate free nitrogen from the atmosphere. These results are at variance with those of P. E. Muller, who held that his own experiments with *Pinus montana* indicated the ability of the tree to assimilate nitrogen from the air if the tree had mycorrhizae on its roots.

The fungi certainly benefit by association with the roots of seedlings. Hansteen-Cranner (6) states that the roots of higher plants excrete various substances, and other investigators have found that

gave these mycorrhizae a hairy appearance. The root was penetrated intercellularly by mycelium as far as the central cylinder, and in a few instances mycelium was found in the central region of the root.

With one exception, the mycorrhizae formed on northern white pine by these four species of fungi were very similar to the corresponding mycorrhizae on Norway spruce, and require no separate description (Fig 6, A and B). *Tricholoma personatum*, however, formed mycorrhizae with hairy mantles rather than the smooth mantles characteristic of the mycorrhizae which it produced on spruce. The bulbous tip of the spruce mycorrhizae also was less evident in the pine mycorrhizae formed by *T. personatum*. On white pine the mycorrhizal mantles developed by all of these fungi were thinner and less compact than those formed on spruce, the

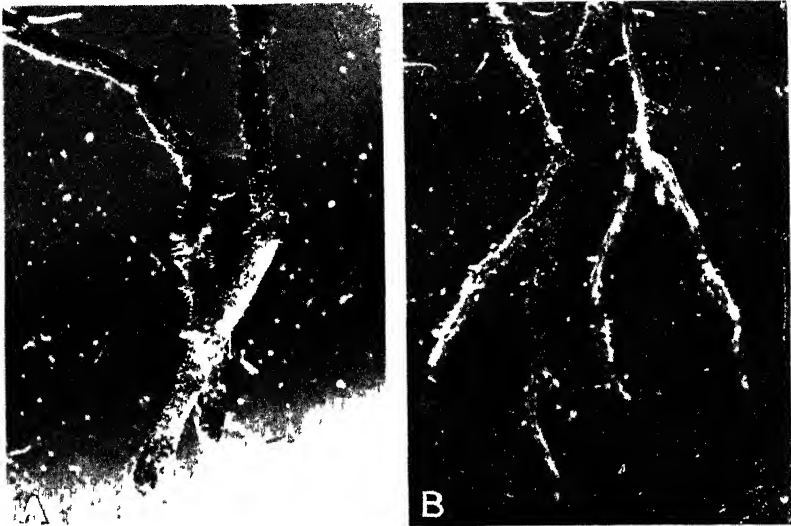


FIGURE 6—A, Early stages of mycorrhiza formation on white pine, in synthesis with *Lycoperdon gemmatum* (nitrate series), B, mycorrhizae on white pine, formed in synthesis with *Tricholoma personatum* (nitrate series)

roots, however, were penetrated by the mycelium just as far in the pine as in the spruce.

#### DISCUSSION

Despite the numerous investigations which have been made there is very little known about the exact way in which mycorrhizae aid or harm the associated higher plants. Experimental evidence of unquestionable character is exceedingly sparse. Much evidence which might be acceptable because of accurate and careful methods must be taken with reservation because of insufficient data, conclusions of considerable importance have been based on experimentation with a very small number of seedlings, and in a few instances pages have been written on the results obtained with only one seedling. Especially when conclusions concerning the physiological significance of mycorrhizae are based on measurement of seedling

them with most of their mineral food substances. If mycorrhizae do not aid the tree in securing these substances, they at least do not appear to hinder the tree in obtaining them unaided.

As evidence of parasitism one may cite the cessation of growth in length of the infected roots and their apparent death after a few months. But if this argument is advanced, one must also be prepared to explain how it is that mycorrhizae can be so prevalent and



FIGURE 7—Mycorrhizae on roots of 4-year-old white pine seedling from a forest nursery

yet not visibly injure the plants. No experimental evidence so far advanced proves beyond doubt that the presence of mycorrhizae is detrimental to trees or seedlings. On the contrary there is considerable experimental and circumstantial evidence to show that the formation of mycorrhizae either is not harmful to the trees, is beneficial to the trees, or is even necessary to the vigorous growth of the plants. The "balance of benefit" theory may not always hold, for

the growth of fungi is accelerated when the fungi are in contact with the roots of plants presumably possessing such excretions. According to Baerthel (1), Wilson found that the development of many species of bacteria is stimulated when they are supplied with root excretions. Melin discovered that the dry weight of fungous mycelium was from 40 to 56 times greater when grown in contact with seeds or seedlings than when grown alone. Another possibility, hitherto unconsidered and as yet unproved, is that the fungi utilize the large amount of organic material which is liberated from the root caps of plants by abrasion. It is not necessary for the fungus to penetrate the root to secure this material. After entering the root, the fungus undoubtedly obtains carbohydrates, especially glucose, and many other substances from the higher plant. Masui found that fungi obtain amino acids, carbohydrates, nitrates, phosphorus, potassium, ammonia, and tannin from the roots. Peklo also thought that fungi might obtain tannin from the roots, whereas Melin declares that the mycorrhizal fungi do not obtain tannin from the roots and are unable to use tannin. The experiments described in this paper indicate that the development of certain mycorrhizal fungi is accelerated by adding to their nutrient media tannic substances extracted from cork.

Irrespective of the precise manner in which the fungi or seedlings individually are aided or harmed by their mutual association, the general effect of the mycorrhizal association is of great importance. Particularly is this true with seedlings in forest nurseries. Kessell (11) reports the failure of a number of nurseries recently started in Australia on new soil. The seedlings in these nurseries germinated but soon turned yellow and were stunted in growth after reaching a height of about 3 inches. Experiments made to determine the cause of these failures failed to demonstrate that season of sowing, watering, shading, commercial fertilizers, pathological conditions, or hydrogen-ion concentration of the soil was responsible for the poor health of these seedlings. At this point a quantity of soil from an old nursery was dressed into the topsoil of the new nursery. After this the seedlings developed normally. The same results were obtained by planting seedlings from an old nursery among the seed beds on the new area. Kessell believes that the absence of the proper fungi to form mycorrhizae is the factor chiefly responsible for the failure of the sowings in the newly established nurseries. Kelley (10) reports a strikingly similar condition in a nursery established on new soil in Pennsylvania. The widespread occurrence of mycorrhizae on the roots of nursery seedlings has been noted generally. In three nurseries the present writer has found abundant mycorrhizae on the roots of practically all seedlings examined (Fig 7). In view of the abundance of mycorrhizae it may be pertinent to inquire if a "normal" seedling can possibly be one without mycorrhizae?

It is the writer's opinion that the presence of mycorrhizae is beneficial to seedlings and probably also to trees in most instances. Although only a part of the total root system of a large tree is in the upper layer of soil where mycorrhizae are found, most of the small roots are in this upper stratum, and in this horizon are also many of the nutrient substances essential to plant life. Many species of trees undoubtedly rely on the roots inhabiting this shallow layer to supply

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it seems illogical to attribute to a fungus ordinarily harmless the death of a seedling or tree that is dying for want of the proper food substances. The death of sickly seedlings has been reported as being due chiefly to fungous activity, but it was not stated that mycorrhizae actually were formed, and unless mycorrhizae were formed it is difficult to see how the presence of mycorrhizae could result in the death of the seedlings.

Finally the writer wishes again to emphasize the importance of basing conclusions on samples sufficiently large. A large number of seedlings must be used in these syntheses in order to eliminate experimental error and to offset the inherent differences present in certain species of trees. Furthermore, mycorrhizae must be present in abundance. The writer believes that large samples can be used if pots, instead of flasks, are employed for the cultures.

#### SUMMARY

Results of a field study of mycorrhizae made in several plantations of conifers include the seasonal occurrence of mycorrhizae, the various types found, the pH values for soil and humus, and a microscopic examination of a very large number of mycorrhizae.

By means of a technic devised for successfully forming mycorrhizae on the roots of seedlings grown under controlled conditions, such formations have been brought about repeatedly on the roots of northern white pine and Norway spruce seedlings in syntheses with *Tricholoma personatum*, *Lycoperdon gemmatum*, *Clitocybe rivulosa* var *angustifolia*, and *C. diatreta*, and these fungi have been definitely established as mycorrhiza formers. Further, eight other species are suspected of forming mycorrhizae on northern white pine and Norway spruce.

Mycorrhizae have been formed in cultures where nitrogen was supplied by inorganic compounds (nitrates) and in cultures where nitrogen was supplied in organic compounds (asparagine, uric acid, glycine, and peptone). Mycorrhizae also have been formed in cultures where no nitrogen was included in the nutrient solution.

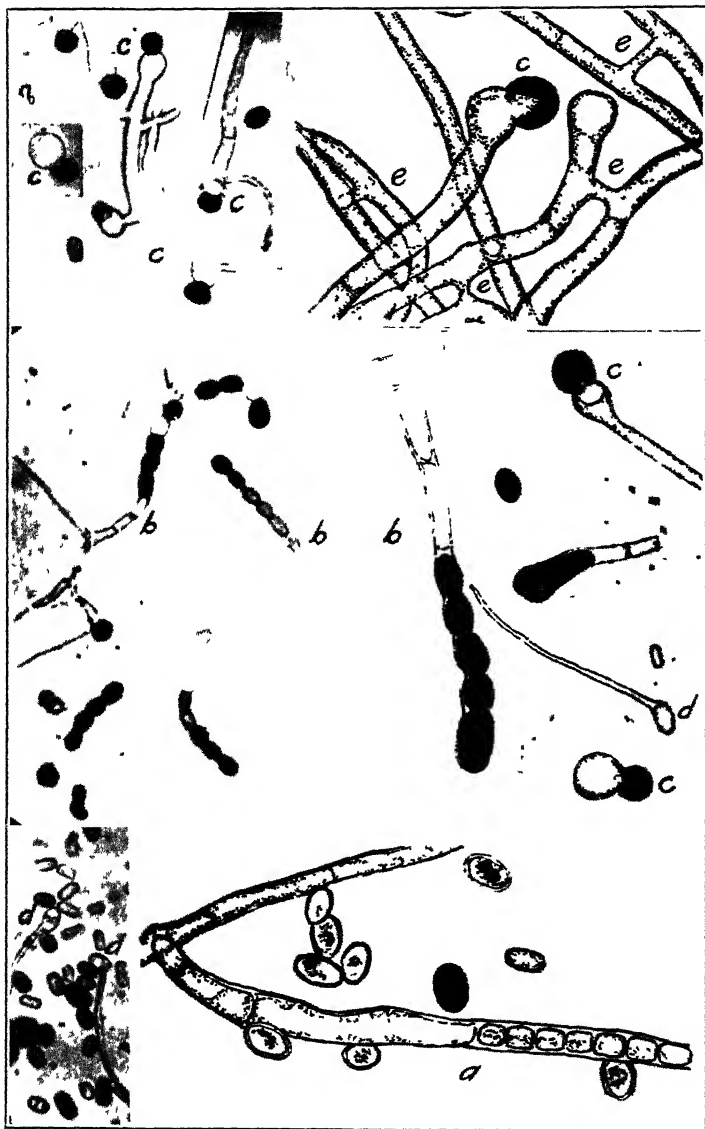
A small amount of evidence was obtained to show that nitrogen is readily assimilated by seedlings without mycorrhizae if the nitrogen is present in inorganic compounds, but that when the nitrogen is present only in organic compounds, especially complex proteins, the seedlings exhibit signs of nitrogen starvation. The presence of mycorrhizae did nothing to alleviate this apparent starvation. Indeed, no conclusive proof was obtained to show that the presence of mycorrhizae on the roots of seedlings is either helpful or harmful to the seedlings.

The formation of mycorrhizae depends on the contact of the right species of fungous mycelium with a growing root tip. No penetration of fungous hyphae was found in older roots. Elongation of a rootlet ceased upon formation of a fungous mantle around its tip. The fungus appeared to be immediately stimulated by contact with the root tip.

Attempts to form mycorrhizae with species of *Penicillium*, *Rhizopus*, and *Mucor* were unsuccessful.

Information was obtained concerning the nutrient and temperature requirements of several fungi which form mycorrhizae.

Fungi held for more than a year in artificial culture did not lose their ability to form mycorrhizae rapidly and in large numbers.



Left, photomicrographs,  $\times$  approximately 200, right, tracings of photomicrographs showing *a*, Microconidiophore with endogenous hyaline conidia, *b*, macroconidiophores with fuscous macroconidia, *c*, macroconidium germinating, *d*, microconidium germinating, *e*, anastomosing hyphae,  $\times$  approximately 400

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# CHROMOSOMES IN GRASS SORGHUMS<sup>1</sup>

By A E LONGLEY

Associate Botanist, Division of Genetics and Biophysics, Bureau of Plant Industry,  
United States Department of Agriculture

## INTRODUCTION

Studies have been undertaken by several investigators to determine whether variations in chromosome number or abnormalities in chromosome behavior during reduction phases are associated with taxonomic differences, unusual genetic behavior, or unexpected results in plant-breeding experiments

The present study of chromosome number in grass sorghums was suggested by the fact that perennial teosinte has twice as many chromosomes as the annual form and its close relative, corn (*Zea mays* L.).<sup>2</sup> It seemed of interest to examine the chromosome number in annual and perennial sorghums, since there are, for example, two species, *Sorghum sudanensis* (Piper) Stapf and *S. halepensis* (L.) Pers., that are hardly distinguishable except that the former is an annual and the latter produces rhizomes and behaves as a perennial. This condition is almost a duplicate of that found in annual and perennial teosinte (*Euchlaena mexicana* Schrad. and *E. perennis* Hitchc.). It seemed possible that a chromosome study of these two and other sorghums might show a difference similar to that found in teosinte.

Plants of 10 different sorghum species were grown in the greenhouses at Washington.<sup>3</sup> The cytological preparations were made from fresh material stained with iron-aceto-carmin solution. The pollen mother cells were collected while undergoing the reduction division phases. One species, *S. versicolor* Anderss., was also studied after the anthers were killed, embedded, sectioned, and stained with Haidenham's haematoxylin.

## JOHNSON GRASS

*Sorghum halepensis* (Johnson grass) sends off rhizomes from the crown which live over winter and start up as new plants the following spring. This species has 20 bivalent chromosomes which are favorably distributed for counting in the prophase of the first reduction division. Figure 1, D, shows clearly the 20 paired chromosomes of *S. halepensis*. Prophases of the two daughter cells of *S. halepensis* showing 20 chromosomes in each cell are illustrated in Figure 1, E.

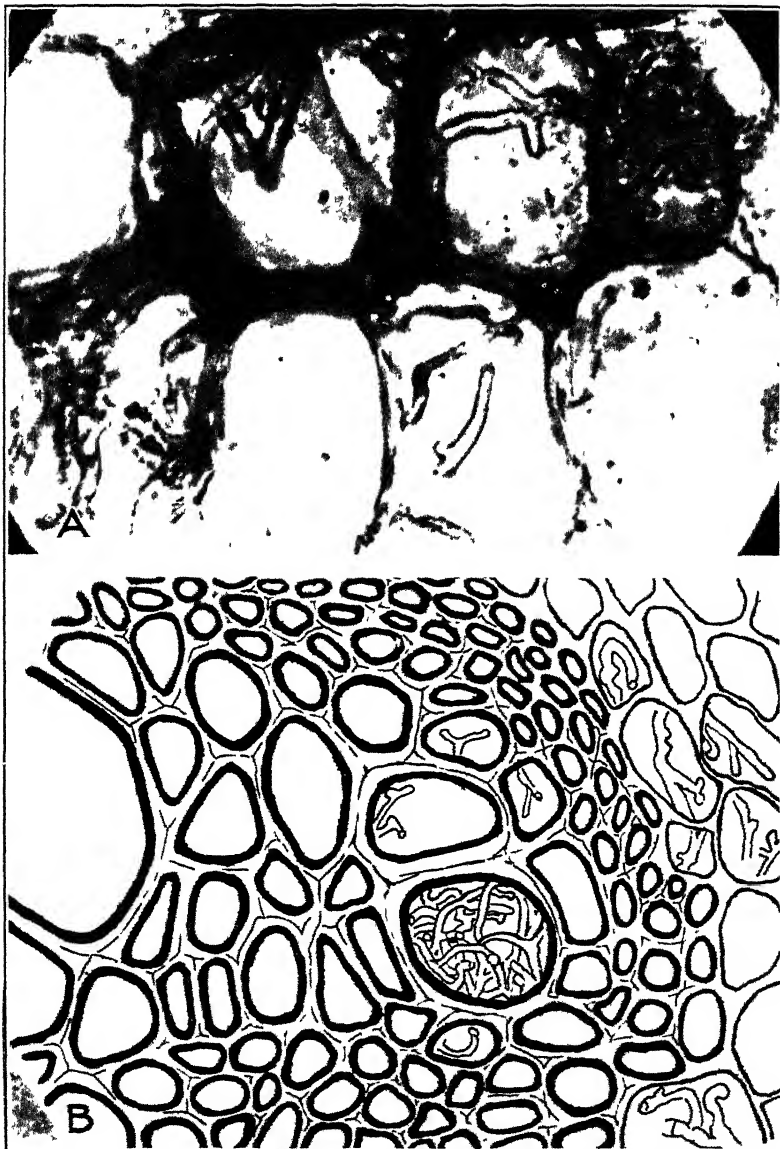
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Seven species of sorghum that do not put out rhizomes from the crown and normally live only one season were found to have 10 bivalent chromosomes in prophases of the first division of the pollen mother cells.

<sup>1</sup> Received for publication July 23, 1931, issued April, 1932.

<sup>2</sup> LONGLEY, A. E. CHROMOSOMES IN MAIZE AND MAIZE RELATIVES. Jour. Agr. Research 28: 673-682, illus. 1924.

<sup>3</sup> H. N. Vinal, of the Division of Forage Crops and Diseases, supplied the seed of the sorghums studied and cooperated in the work as it progressed.



A, Photomicrograph of hypha in parenchyma of petiole,  $\times$  approximately 320, B, tracing from a photomicrograph of transverse section of petiole, showing hyphae in tracheal tubes and in parenchyma,  $\times$  approximately 160

of this species. Material from this species was unusually favorable material for chromosome study, the chromosomes are large, the number small, and many of the second-division spindles showed the five V-shaped chromosomes going to the poles in an almost stereotyped manner.

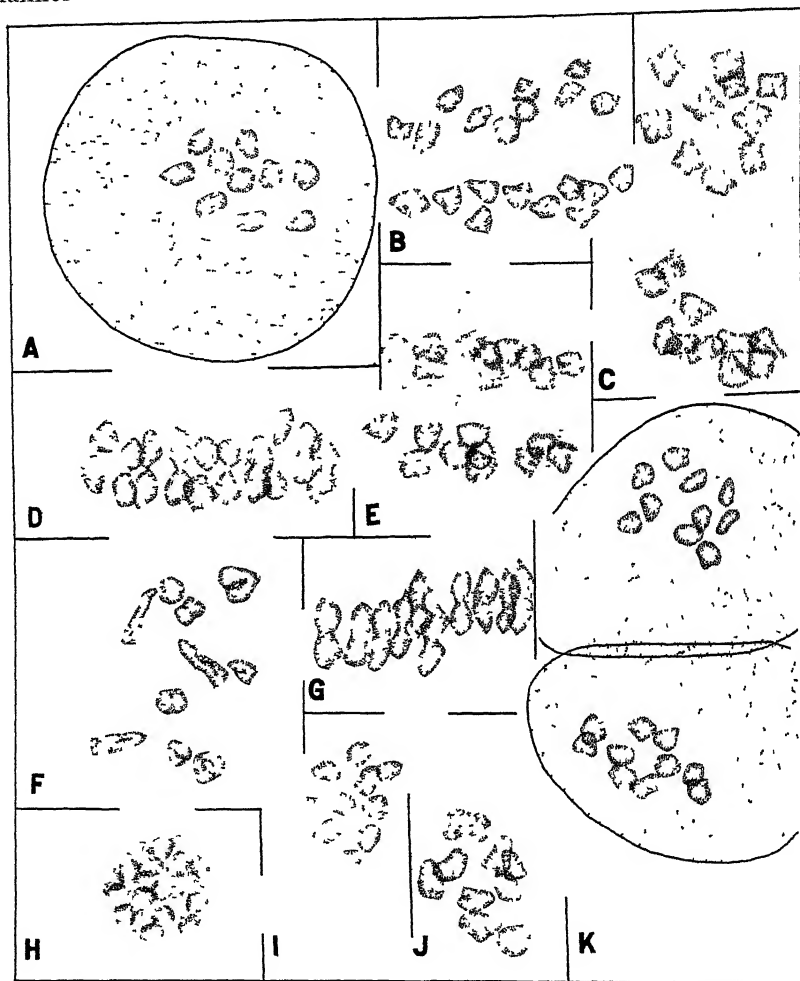


FIGURE 2.—Chromosomes in pollen mother cells of sorghums,  $\times 1,500$ . A, B, and C, first division phases in *Sorghum verticilliflorum*. D and F, first-division metaphase and anaphase in *S. virgatum* (Hack.) Stapf. E and G, diakinesis and first-division metaphase in *S. drummondii* Nees. H, first-division metaphase in *S. purpureo-sericeum* (Hachst.) A. Schers. and Schweinf. I, first-division metaphase in *S. heurisma*. J, first-division metaphase in *S. arundinaceum* (Willd.) Stapf. K, second-division metaphase in *S. arundinaceum*.

The haploid chromosome number of *Sorghum purpureo-sericeum* was found to be 20, as seen in diakinesis of the pollen mother cells. Figure 2, H, shows a typical first-division metaphase in which 20 chromosomes can be readily counted. This annual representative of the genus *Sorghum* is an exception to the usual chromosome condition in this genus, in which, with this exception, all annual forms were found to have 10 or less as their haploid chromosome number.

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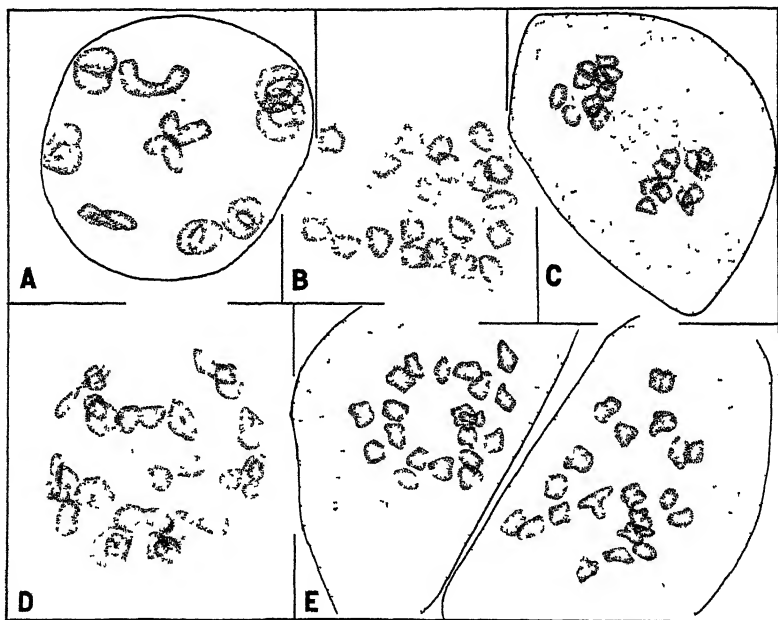


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An eighth annual sorghum species, *Sorghum versicolor*, a short-lived annual coming from southeastern Africa, was found to have five as its haploid chromosome number. Figure 3, A-F, shows six characteristic phases in the reduction divisions of the developing pollen mother cells

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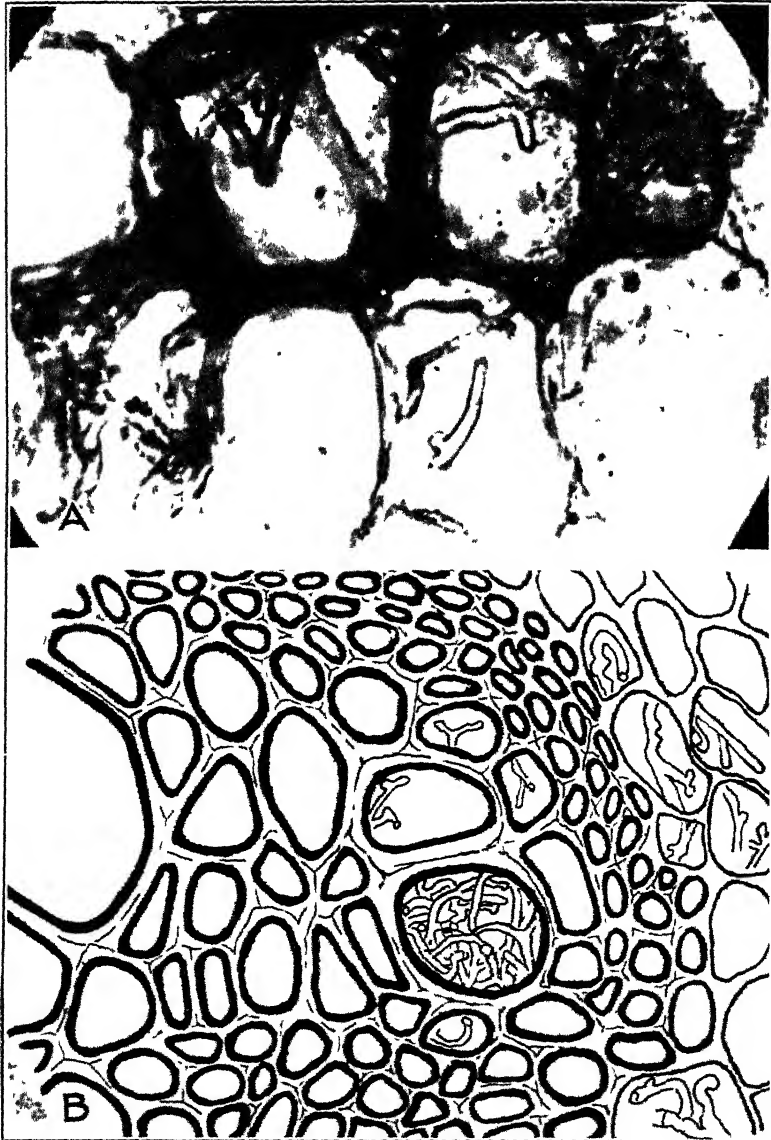
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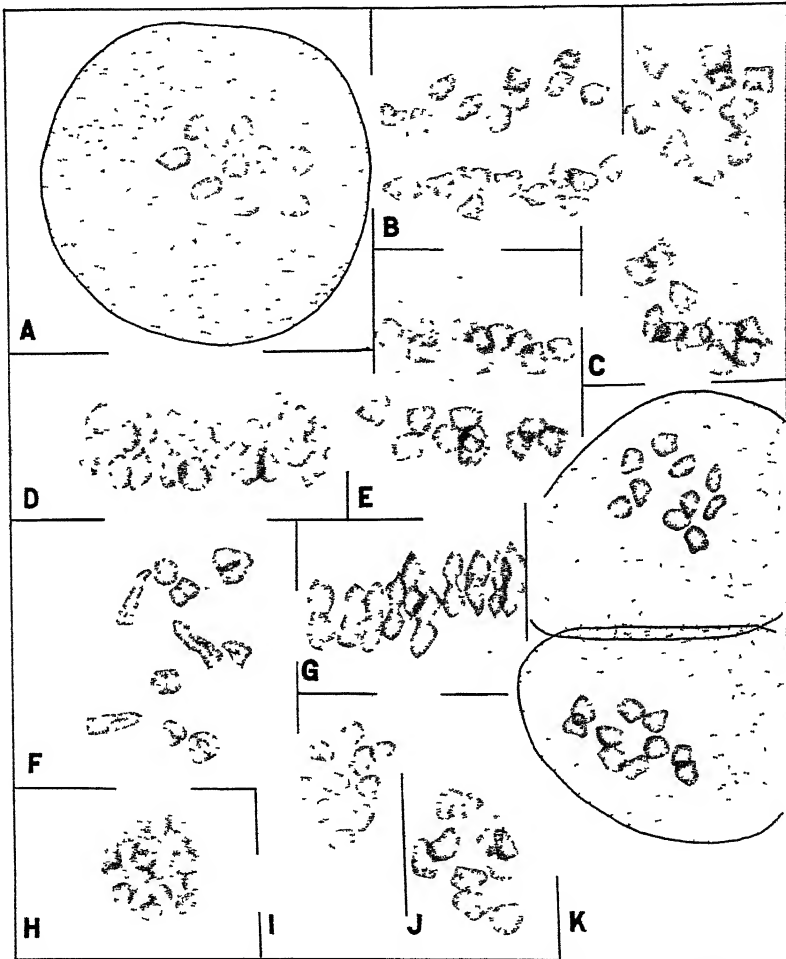


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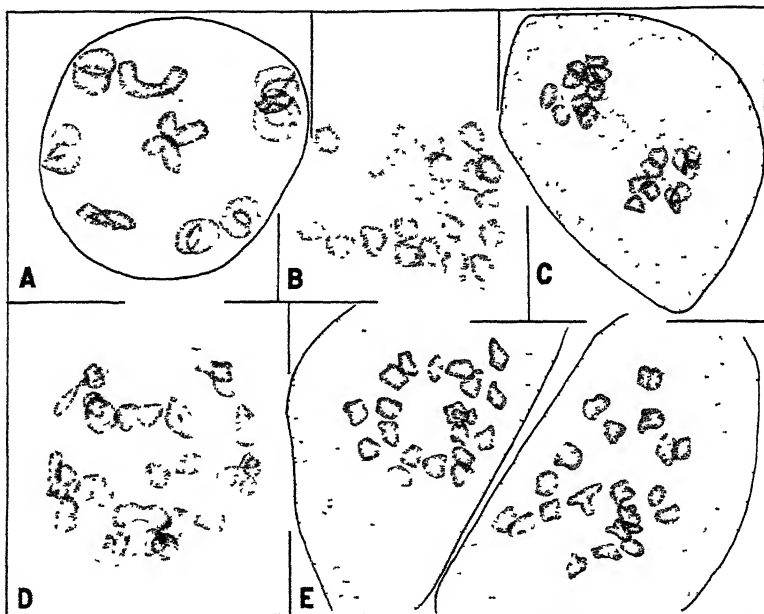


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The habit of this species is that of an annual, and the chromosome number is that found in most of the annual grass sorghums

#### SORGHASTRUM NUTANS, CLOSELY RELATED TO SORGHUM

*Sorghastrum nutans* (L.) Nash is a species that taxonomically is not far removed from the sorghums. Church<sup>6</sup> has found 20 to be the reduced chromosome number of this perennial grass, which is the number found in perennial sorghum species

#### OTHER GENERA OF ANDROPOGONEAE

Among those genera of the tribe Andropogoneae which are more distantly related to sorghum the analogy in chromosome numbers is not so close. In *Erianthus*, Bremer<sup>7</sup> found 30 to be the haploid chromosome number of four species. *Miscanthus sinensis* var *zebrinus* Beal was found by Church<sup>8</sup> to have 21 chromosomes in gametic cells. *Andropogon ischaemum* L., according to Kuwada,<sup>9</sup> has 34, and *A. scoparius* Michx. and *A. furcatus* Muhl. have, respectively, 55/2 and 34, according to Church, as their haploid chromosome numbers. In the genus *Saccharum* the lowest chromosome number reported is that of *S. narenga* (Ham.) Wall., which Bremer gives as 15. Other species and species hybrids with chromosome numbers ranging from 20 to more than 100 have been studied by Kuwada and by Bremer. All plants of these five genera are perennials, and the lowest chromosome number found was 15, so that nothing contradictory to the relationship between high chromosome number and perennial habit is encountered in this group.

Exceptions similar to that found in *Sorghum purpureo-sericeum* were found in species from the more distantly related genera. These exceptions are *Imperata cylindrica* (Lam.) Beauv., which Bremer<sup>10</sup> found had 10 as its haploid chromosome number, and *Cymbopogon nardus* (L.) Rendle, in which Kuwada<sup>11</sup> found this same low number. Both of these species are perennial, and unless there exist related annual species with five chromosomes, the relationship found between high chromosome number and the perennial character is not a rule that applies to all genera of the tribe Andropogoneae.

The association between low chromosome number and the annual habit finds but one exception among the sorghum species and only a few exceptions in the closer relatives that have been examined cytologically. The chromosome condition is identical with that found in *Euchlaena* and its relatives, in which the perennial species has double the chromosome number of the annual form.

#### CONCLUSION

If the chromosomes of the perennial sorghums represent a duplication of the chromosome set found in annual forms, as seems to be the case in perennial teosinte, it indicates that the perennial plants have been derived from annual ancestors having 10 chromosomes. Whether this be the case or not, chromosome number is a character that should be of considerable value in the classification of the sorghums.

<sup>6</sup> CHURCH, G. L. MEIOTIC PHENOMENA IN CERTAIN GRAMINEAE, II PANICEAE AND ANDROPOGONEAE. Bot. Gaz. 88: 63-84, illus. 1929.

<sup>7</sup> BREMER, G. THE CYTOLOGY OF THE SUGARCANE. Genetics 7 [293]-322, illus. 1925.

<sup>8</sup> CHURCH, G. L. Op. cit.

<sup>9</sup> KUWADA, Y. Op. cit. (See footnote 4, second citation.)

<sup>10</sup> BREMER, G. Op. cit.

<sup>11</sup> KUWADA, Y. Op. cit. (See footnote 4, second citation.)

## DISCUSSION

## THE GENUS SORGHUM

The outstanding result of this determination of chromosome numbers in a few representative grass sorghums is that the perennial Johnson grass has double the number of chromosomes found in the annuals. This difference between Johnson grass and Sudan grass in chromosome number was also found in other annual sorghum species available for study. In all but two a haploid chromosome number of 10 was found in the annual forms and double this number in the perennial form. The two exceptions are *Sorghum purpureo-sericeum*, which has 20 chromosomes, and *S. versicolor*, which has only five

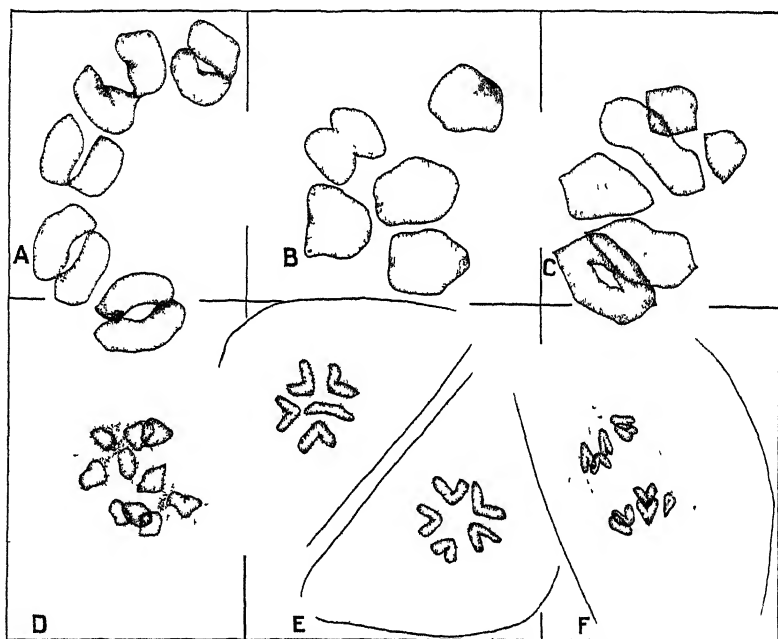


FIGURE 3.—Division phases in pollen mother cells of *Sorghum versicolor*. A, Diakinesis, B and C, first division metaphases, early and late, D, first division anaphase, E, second division metaphase, F, second division anaphase. (A, B, and C,  $\times 1,000$ ; D, E, and F,  $\times 1,000$ )

It is probably a coincidence that the decrease in chromosome number is usually found associated with a reduction in life span. *S. versicolor* is a shorter-lived annual than any of the forms with 10 or more chromosomes.

Kuwada<sup>4</sup> was the first to study the chromosomes in sorghum. He found that *S. vulgare* Pers has 10 as its reduced number. Marchal<sup>5</sup> found the same number for this species, and the writer found no deviation from this number in any of the following varieties: Blackhull kafir, Dwarf Shantung kaoliang, feterita, Sumac sorgho, White kaoliang, Black Amber sorgho, Dwarf Yellow milo, and Dwarf White milo.

<sup>4</sup> KUWADA Y. ÜBER DIE CHROMOSOMENZAHLE VON ZEA MAYS L. Bot Mag [Tokyo] 29: 83-89, illus. 1915.

— DIE CHROMOSOMENZAHLE VON ZEA MAYS L. Jour Col Sci, Imp Univ Tokyo 39, art. 10, 148 p., illus. 1919.

<sup>5</sup> MARCHAL, E. J. RECHERCHES SUR LES VARIATIONS NUMÉRIQUES DES CHROMOSOMES DANS LA SÉRIE VÉGÉTALE. 105 p., illus. Bruxelles [1920].

# THE GROWTH CURVE IN BARLEY<sup>1</sup>

By MERRITT N POPE<sup>2</sup>

*Agronomist, Division of Cereal Crops and Diseases, Bureau of Plant Industry,  
United States Department of Agriculture*

## INTRODUCTION

When an investigation of the catalase activity in relation to the growth of barley was initiated it was found to involve a comprehensive study of the growth curve of the plant. The results of the growth studies proved to be of sufficient interest to warrant a separate paper on this aspect of the original problem. A second paper deals specifically with catalase activity in relation to the growth curve (17')<sup>3</sup>

## MATERIAL AND METHODS

Plants on which growth measurements were taken were grown at the Arlington Experiment Farm, Rosslyn, Va. Two varieties of barley were sown: Hannchen, C I<sup>4</sup> No 531, a 2-rowed spring type that stools abundantly, and Tennessee Winter, C I. No 3546, a 6-rowed variety that stools less freely. One plot of each variety was sown on March 14, 1928. The soil in these plots was fairly good but was subject to considerable erosion.

Daily samplings for growth measurements were begun with Hannchen on March 28 and with Tennessee Winter on March 30 and were continued to June 4. After this date the interval between samplings was lengthened to two or more days because of the shortage of suitable material. At the beginning the daily samples, consisting of 20 plants, were all taken from one end of the plot, working toward the other end on succeeding days. The plots soon showed considerable variability, so the most nearly uniform part of each plot was divided transversely into six parts of approximately equal area, and the same number of plants were taken, as they came, from each division to make up the daily samples. On April 17 variability was further reduced by thinning the plants 2 to 3 inches apart in the drill row and by rejecting abnormally small ones.

Since it is impossible to obtain all the roots of a field-grown barley plant, the present investigation was made upon that portion of the plant above the seed. Later, when the permanent roots appeared, the portion of the stem above them was used. The tops varied in weight with the number of tillers per plant, so the main-shoot measurements only were made the basis of this study, as the main shoots varied less than the entire plants. The variation in the weights of individual plants as compared with that in the weights of their main shoots is shown in Figure 1.

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<sup>2</sup> The writer acknowledges his indebtedness to Dr. C. O. Appleman, of the University of Maryland, for many helpful suggestions and criticisms.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 340.

<sup>4</sup> C I. No. refers to accession number of the Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.



barley Variations in amount of growth from the ideal may be expected and when present are reflected in the curve of growth. A different succession of weather changes would produce a slightly different curve, as is indicated by the work of Gregory (5), who, in studying the effect of environment on the growth of barley, found a high positive correlation between day temperature and both net assimilation rate and efficiency index, and a significantly positive correlation between day temperature and relative leaf growth. The correlation between night temperature and these three indicators of growth was significantly negative in each case. Relative leaf growth was highly and negatively correlated with radiation, and efficiency index was independent of radiation.

Total height, or the length from crown to farthest leaf-tip extension, indicates fairly well the growth stage of a plant. As a measure of growth it is, however, subject to certain sources of error. The leaves of a grass are alternate, and growth occurs in the basal region of the leaf. Each leaf has its own grand period of growth, and therefore the grand period of growth, as indicated by the curve of the height of the plant,

varies with the growing activity of the leaf extending the greatest distance distally from the crown. The younger leaf will be past its early slow-growing stage when it pushes out beyond the tip of the next older leaf which has just about finished its period of rapid growth. If the height of a single plant is measured at intervals short enough, such as are shown in Figure 2, the curve of growth will show a succession of accelerations corresponding to the appearance of the tip of young, actively growing leaves. This error is greatly reduced by averaging the measurements of a plant population which varies in number and stage of growing leaves. It is clearly present, however, in the height-growth curves at about the time of endosperm exhaustion. At this time the seedlings were very nearly in the same stage, the first leaf nearly at maximum length and the second leaf, while actively growing, needing several days' growth to equal the first in length.

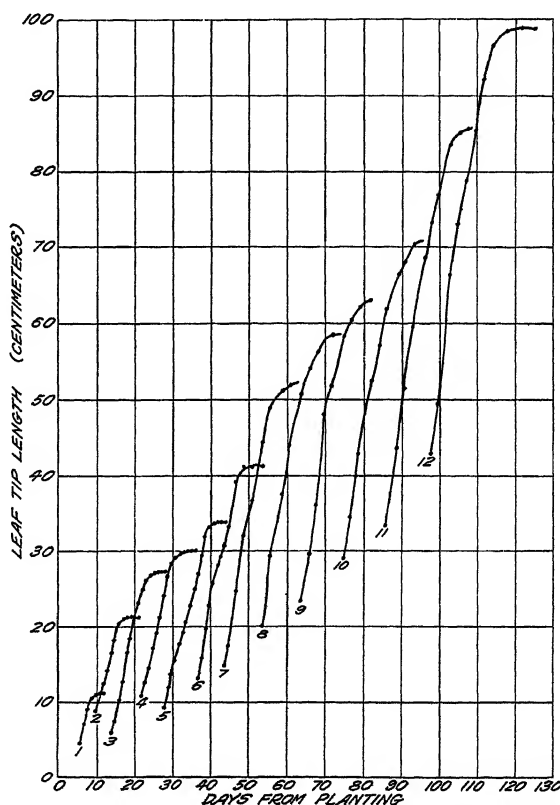


FIGURE 2.—Length-growth curve of plant No. 50, Arlington greenhouse, 1930, showing measurements of each successive leaf from its appearance until elongation ceased

The plants were dug or pulled, wrapped in moist cheesecloth, and taken to the laboratory. The main shoots were then removed, placed again in moist cheesecloth, and kept there until they were measured and weighed. Abnormally small plants without vigorous tillers were discarded. The length in centimeters of the main shoot from seed or crown to the extreme leaf tip and the green weight of the main shoot were determined for each plant. While the plants were small, representative shoots were bulked and dried to constant weight in a vacuum oven at 80° C. The average dry weight per plant was then calculated from the average green weight. As the plants became larger all the main shoots in a day's sample were clipped into approximately  $\frac{3}{4}$ -inch pieces and mixed thoroughly. Determinations

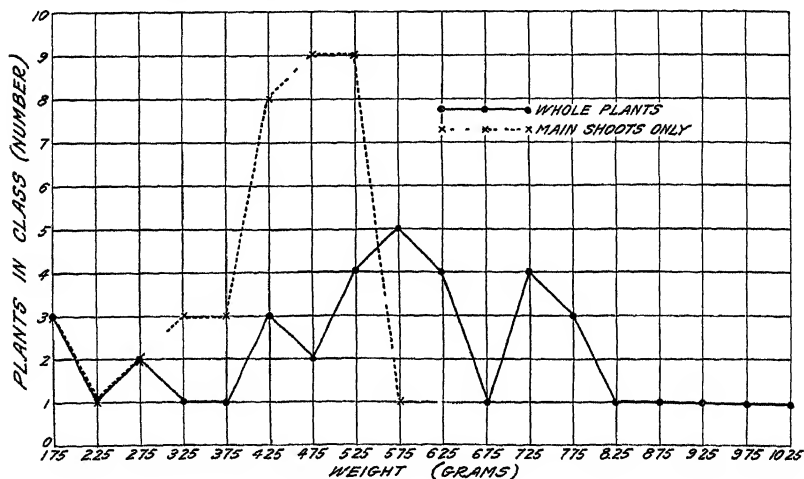


FIGURE 1.—Frequency distribution comparing the variability in green weight of whole plants with that of their main shoots. (Data from greenhouse population of a single day)

of dry matter were made on duplicate 5-g.<sup>5</sup> samples, or even on 10-g.<sup>6</sup> samples when the plants became still larger.

By the eighty-fifth day most of the kernels had started to develop. Beginning on this day separate measurements of both length and weight were made on the culm and the spike. These measurements made possible a study of the growth of the shoot exclusive of the kernels.

Notes were also taken on the condition of reserve foods in seed endosperm, leaf stage, presence and stage of development of seminal and permanent roots, presence of tillers, appearance of boot leaf, emergence and length of awns, emergence of spike, color and dehiscence of anthers, age of developing kernel, browning of awn tips, browning of glumes, and cessation of translocation in the kernels as shown by degree of drying.

#### FACTORS AFFECTING RELIABILITY OF THE DATA

An ideal experiment on growth would be one conducted under temperature and moisture conditions exactly optimum at all times. This study was made upon plants grown under conditions of about normal temperature and slightly subnormal but sufficient rainfall. On the whole the climatic conditions were rather favorable for spring-sown

<sup>5</sup> g is the abbreviation recently adopted by the Government Printing Office for grams.

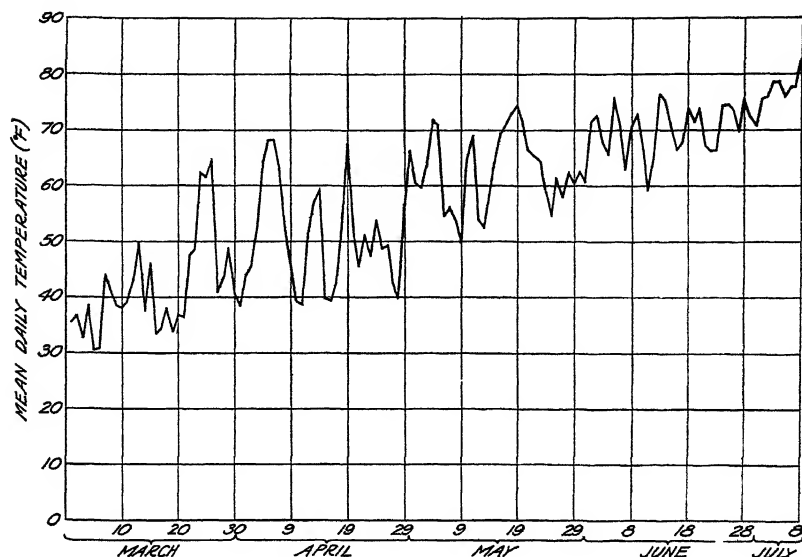


FIGURE 3—Mean daily temperature (in ° F) from March 1 to July 8, 1928, at the Arlington Experiment Farm, Rosslyn, Va

TABLE 1—Summary of weather conditions from March 1 to July 9, 1928, at Washington, D C

Item	March	April	May	June	July (1-9)
Dates of killing frosts.....	19, 20, 31	16, 17, 18	(a)	(a)	(a)
Accumulated excess (+) or deficiency (—) in temperature since first day of month..... ° F..	+49	—42	—23	—40	+28
Excess (+) or deficiency (—) in precipitation this month as compared with normal..... inches..	—1 58	+1 25	+0 31	—1 47	—1 16
Accumulated excess (+) or deficiency (—) since Jan 1..... inches..	—2 82	—1 57	—1 26	—2 47	—3 63
Mean percentage of relative humidity					
8 a m.....	74	67	74	79	72
Local noon.....	55	49	51	62	52
8 p m.....	54	54	60	70	63
Normal percentage of relative humidity					
8 a m.....	68	68	69	74	<sup>b</sup> 78
Local noon.....	51	48	50	56	<sup>b</sup> 57
8 p m.....	55	54	58	66	<sup>b</sup> 73
Mean percentage sunshine.....	60	55	62	53	85
Normal percentage sunshine.....	51	58	61	62	<sup>b</sup> 64
Total hours possible sunshine.....	371 1	397 4	444 0	446 0	133 3

<sup>a</sup> None

<sup>b</sup> For the month

## DEVELOPMENTAL STAGES OF THE BARLEY PLANT

Morphologically there are certain definite stages in the development of the barley plant. These stages and the plant age at which they occurred in this study are noted in Table 2. The placing of these stages in the growth curve should aid in interpreting the latter.

Another source of error is the fact that neither the absolute nor the relative height of a plant variety is constant for different environmental conditions. This was shown by Harlan (?) Abyssinian barley was tall at Chico, Calif., and very short at St. Paul, Minn., Williston, N. Dak., and Moccasin, Mont. Odessa barley was tall at Williston and Moccasin and very short at St. Paul, and stood eighth on the list of 13 varieties at Chico. This error, due to varietal variation, is at least partially overcome by sowing the two types of barley.

The use of green weight in measuring growth also is subject to several errors. At the stage characterized by rapid increase of dry matter there may be an actual loss of water, causing the green weight to remain approximately stationary. As the plant nears maturity the moisture content decreases and the green weight approaches the dry weight in value. At this stage green weights indicate little or no growth, when actually that process is progressing rapidly. Green weight during active growth also varies according to the tissue turgidity, which in turn depends on the available soil moisture and the air moisture. Small unremoved drops of rain or dew on the plant at sampling time also introduce error.

Deposition, or at least translocation, of solids in the plant continues until growth ceases and the plant parts die or become dormant. Dry weight is the most reliable measure of growth and is emphasized in this study. Even this standard is not, however, free from error. At all stages of development and growth, parts of the plant are liable to mechanical and pathological injuries which are not always recognizable. This is especially true during the maturing phases, when the leaves gradually die, they are also leached by rain and may be broken off by wind. The situation is still further complicated by the fact that from flowering to maturity the developing seeds (which are new plants parasitic on the parent) were weighed with the spike, since it was impracticable to separate the grain from the seed coats, the latter being maternal tissues which it is practically impossible to remove from the caryopsis. An approximation of the dry weight of the shoot without the seeds was reached by adding to the average dry weight of the culm the average dry weight of the spike on the day of flowering. This is recognized as only an approximation but is considered rather close, as the growth of the maternal tissues in the spike probably is very nearly completed at this stage.

#### WEATHER CONDITIONS DURING THE GROWING PERIOD

Mean daily temperatures for the period from March 1 to July 8, 1928, computed with the aid of the planimeter from thermograph records, are shown in Figure 3, and amount and distribution of rainfall in Figure 4. A summary of the weather conditions for the same period in Washington, 2 miles away, is given in Table 1.

The cool weather, especially in June, allowed an almost normal development of the plants. This cool weather, together with a slight deficiency in rainfall, was unfavorable to such plant diseases as are caused by *Helminthosporium* and rust, which often result in serious injury to spring-sown grains in Virginia.

TABLE 2—*Morphological stages of development in the barley plant, and the plant age at which these stages occurred at the Arlington Experiment Farm, Rosslyn, Va., in 1928*

Stages of development	Hannchen variety		Tennessee Winter variety (spring-sown)	
	Date	Plant age	Date	Plant age
		<i>Days</i>		<i>Days</i>
First leaf.....	May 29	15	May 31	17
Second leaf.....	Apr 5	22		
Endosperm nearly exhausted.....	Apr 8	25	Apr 5	22
Scutellum shriveled.....	Apr 10	27	Apr 7	24
Third leaf.....	Apr 16	33	Apr 17	34
Crown well defined.....	Apr 17	34	do	34
Crown roots started.....	do	34		
Tillers first appear.....	Apr 22	39	Apr 20	37
Fourth leaf.....	Apr 24	41	Apr 21	38
Tillers well developed.....	May 2	49	May 2	49
Jointing.....	May 18	65	May 18	65
Basal leaves mostly dead.....	May 21	68	May 21	68
Boot leaf showing.....	May 31	78	May 30	77
First awns emerging.....	June 2	80	June 2	80
First flowering.....	June 7	85	June 7	85
Tip of boot leaf dying.....	June 17	95		
Awns brown at tip.....			June 19	97
Spikes nearly dried out.....	July 2	110	July 2	110

## EXPERIMENTAL DATA

### GROWTH IN LENGTH

The length-growth curves of the two varieties, Tennessee Winter and Hannchen, are given in Figures 5 and 6. These curves show that the first leaf of the plants grew rapidly in length up to April 8, when the endosperm was almost exhausted. Growth of the crown roots was first evident on April 17, coincident with a second increase in rapid elongation. It is true that this second increase followed a 3-day period of relatively high temperatures, but from a study of the large-scale graph (fig. 9) of the dry weights of the two varieties, it would seem that temperature as a factor might be disregarded, since Hannchen showed an inflection in the curve at this time.

About April 21 there began another period of slower growth. The temperatures during this period were neither very high nor very low. First tillers had appeared but had not yet developed good roots. By April 21 the tiller roots in Hannchen were developing well and the curve again started upward. This increase, however, again was accompanied by higher temperatures, so that here, too, it is difficult to determine whether the increase in growth rate was due to additional root tissue or to higher temperatures. Leaf length began to taper off rapidly in Tennessee Winter on June 7, when first flowering occurred, the awn-tip length tapering off on June 17. In Hannchen flowering occurred on June 7, but the leaf length tapered off much more slowly, and the awn-tip length grew rapidly until June 17.

With both the barley varieties used in these experiments the results differ from those of Van de Sande-Bakhuyzen and Alsberg (21) with wheat, who found an inflection of the curve of length occurring at flowering. One factor contributing to this difference may be that wheat flowers at a later stage of elongation than does barley.

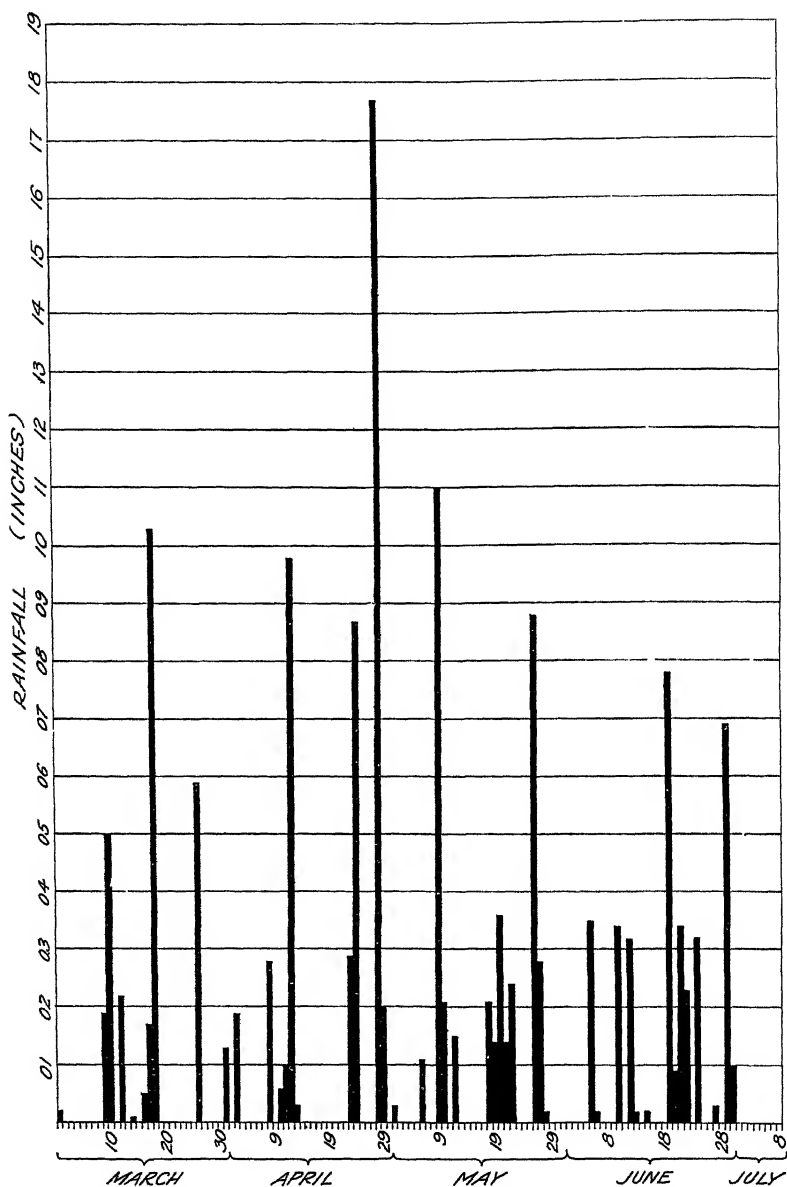


FIGURE 4—Rainfall (in inches) from March 1 to July 8, 1928, at the Arlington Experiment Farm, Rosslyn, Va

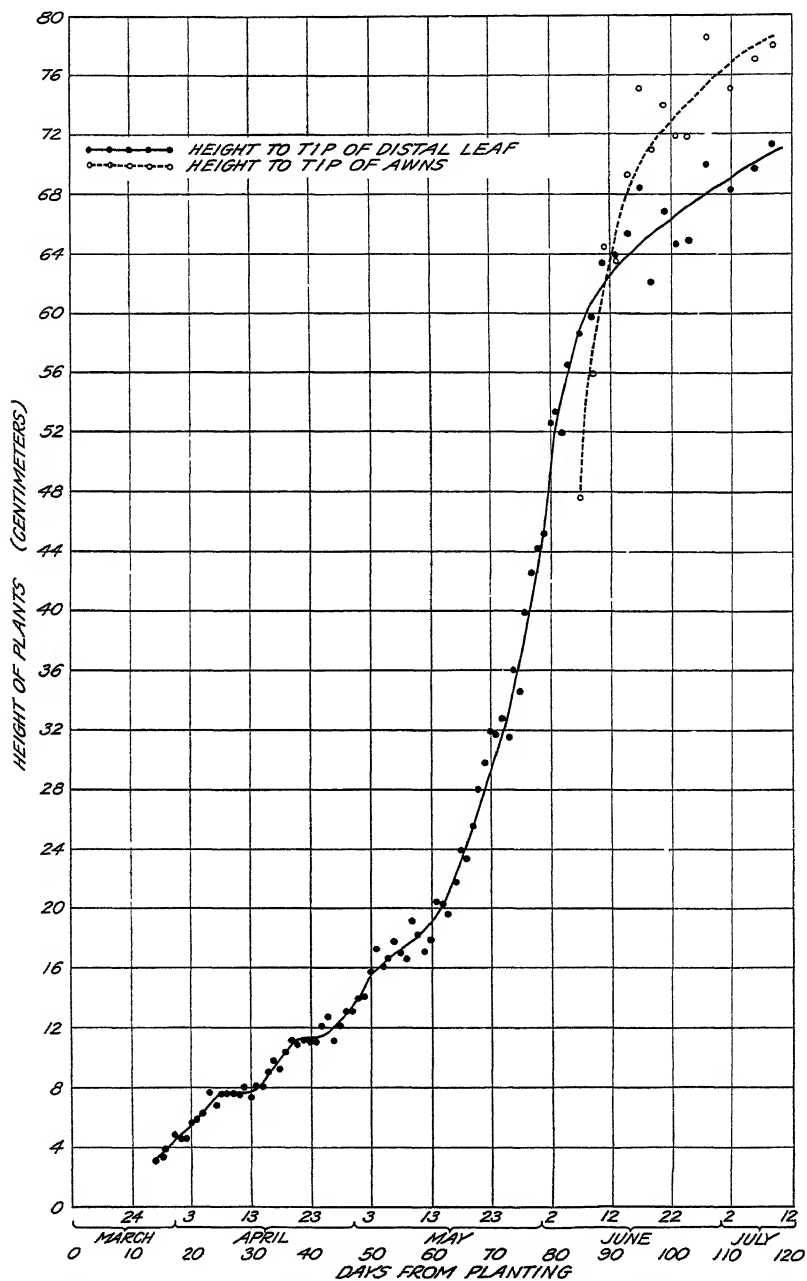
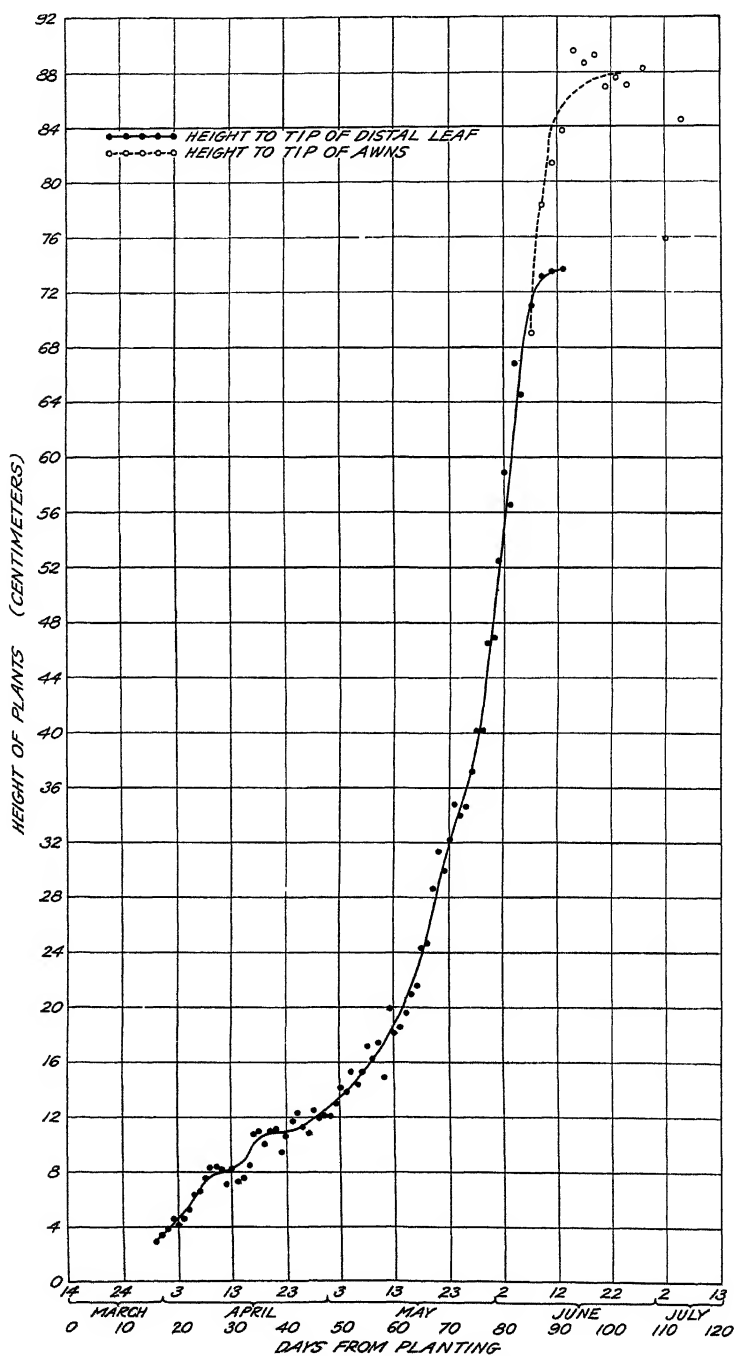


FIGURE 6.—Growth in length of spring-sown Hannchen barley from emergence to maturity



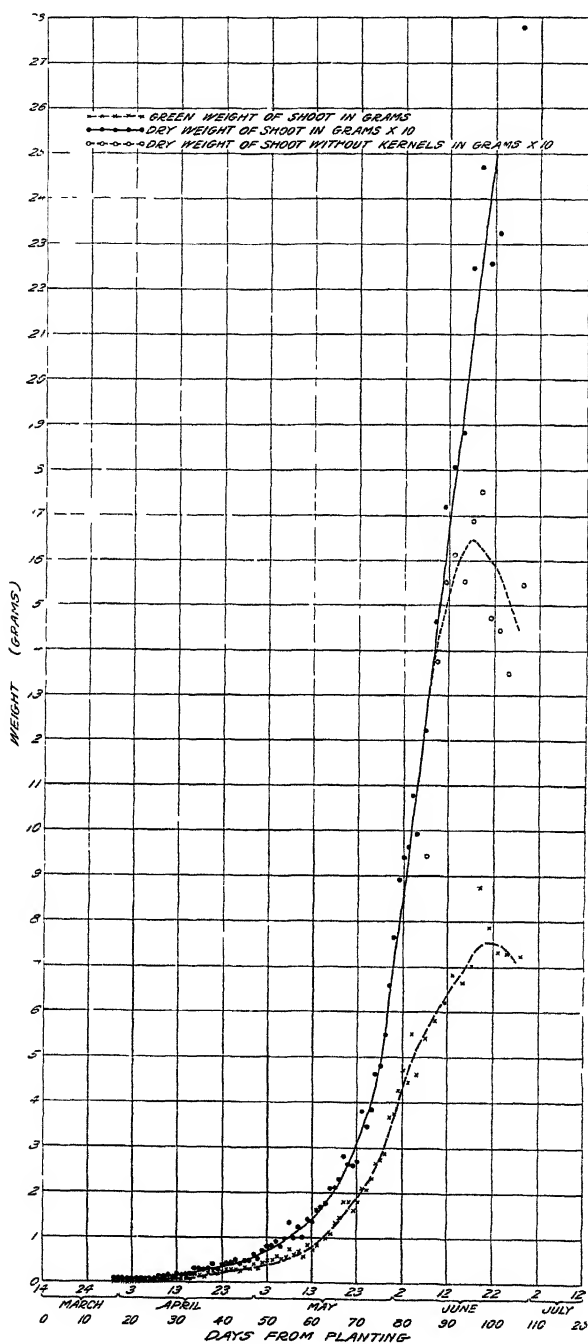


FIGURE 7—Growth in average green weight of main shoot, dry weight of main shoot, and dry weight of main shoot, with kernels excluded, of spring-sown Tennessee Winter barley from emergence to maturity

## GROWTH IN WEIGHT

The daily growth in weight of Tennessee Winter is shown in Figure 7. This curve is comparatively smooth. Prior to May 2 the curves of both green and dry weights had risen only slightly above the base line. During this period length-growth rate of the shoot varied with stage of development much more than did weight-growth rate. However, a larger scale graph (fig. 9) shows a tapering off in weight-growth rate as well as in length-growth rate coincident with exhaustion of the endosperm (second-leaf stage). Throughout this early period there is evident a gradual increase in weight-growth rate, which reached its maximum on May 24, shortly after jointing began.

First flowering occurred on June 7, and after this date the green and dry weights of culm and spike were determined separately. In order to correct for kernel weight, the dry weight of the spike of June 7 was added to the dry weight of the culm on each of the days following that date. The sum of these two weights was the weight of the shoot. This procedure is believed to be justified, since the spike tissue, excluding the kernels, had very nearly reached its maximum mass on June 7. Any error occurring probably would be on the side of less weight.

It will be seen from the curve that when weights of the shoots minus the kernels are plotted as a continuation of the shoot curve before June 7, no perceptible inflection occurs until June 11. This again fails to agree with the results of Van de Sande-Bakhuyzen and Alsberg (21) for the length-growth curve of wheat. The weight of the spike alone started an abrupt increase on June 7, as would be expected owing to the rapid increase in kernel mass. The weight of the culm alone increased at an equally rapid rate until June 11, tapered off slightly on June 13, and after a drop on June 15 climbed again until June 19. After this date leaching by rain and leaf breakage caused a decided drop. As was found by Burd (2), the total weight of the shoot plus kernels increased with almost no change in the direction of the curve to June 19 (97 days), when the awns commenced to turn brown at the tips. From this time on the plants rapidly turned brown and the kernels finally dried out, being almost dry and waxy on July 2.

The growth in weight of Hannchen (fig. 8) differed somewhat from that of Tennessee Winter. In the early part of the growth period (up to April 7) Hannchen was generally heavier in both green and dry weights, from April 8 to 16 it was almost the same, but from April 18 to maturity it was outstripped by Tennessee Winter. Final green and dry weights were much smaller for Hannchen. However, similar changes in the growth curve, as shown in Figure 9, occurred at very nearly the same points as in Tennessee Winter. There was an increase in rate of growth after May 2, when tillering became active, and a further acceleration beginning about May 22, shortly after jointing had been initiated.

In determining the developmental stage of the plant a rough average of 20 plants was used. Temperature as a factor in modifying the curves in the period from April 17 to May 3 may be disregarded, since the inflections were initiated on different dates in the two varieties (fig. 9) and seem to have no relation to a variation in that period of about 29° F. in mean daily temperature. In order to hook up the stage of development more definitely with the growth curve in this early period of growth, the curve of early weight growth in Hannchen



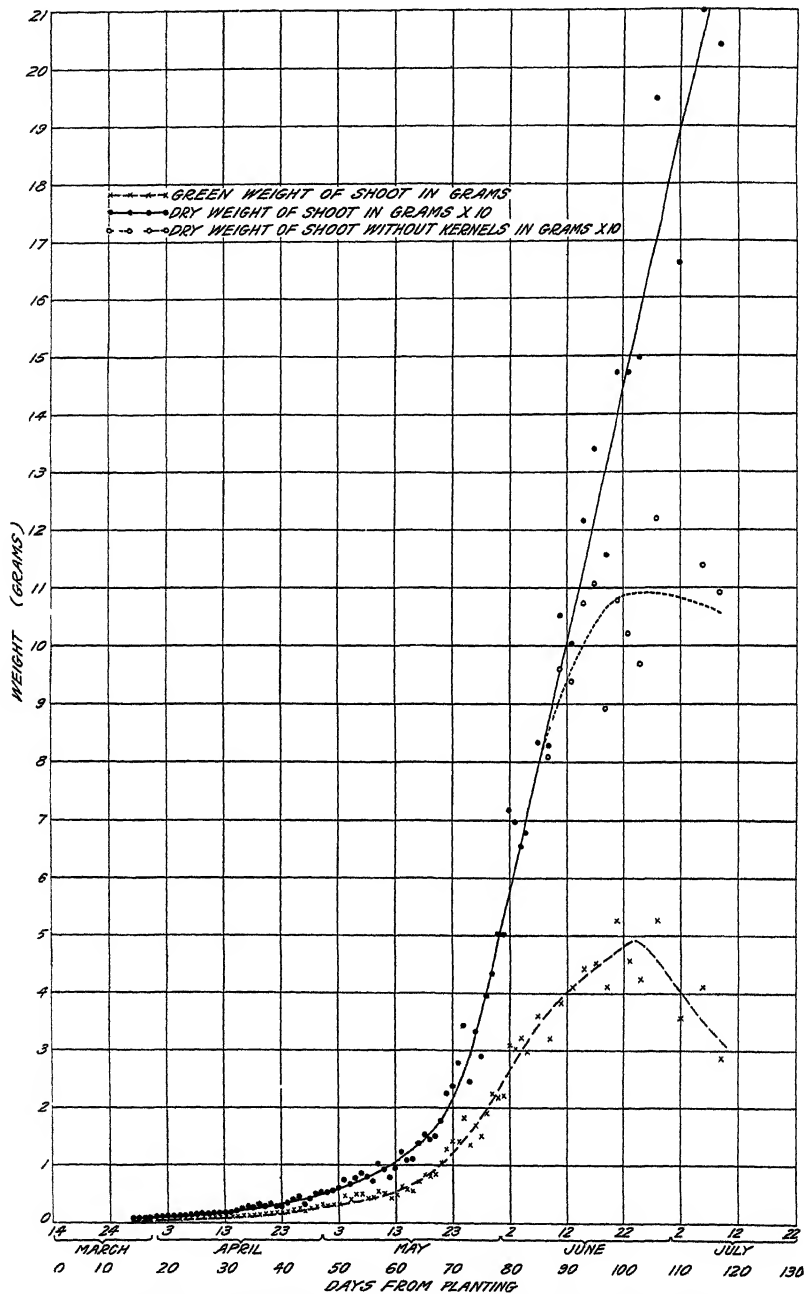


FIGURE 8—Growth in average green weight of main shoot and in dry weight of main shoot with kernels excluded of spring-sown Hannechen barley from emergence to maturity

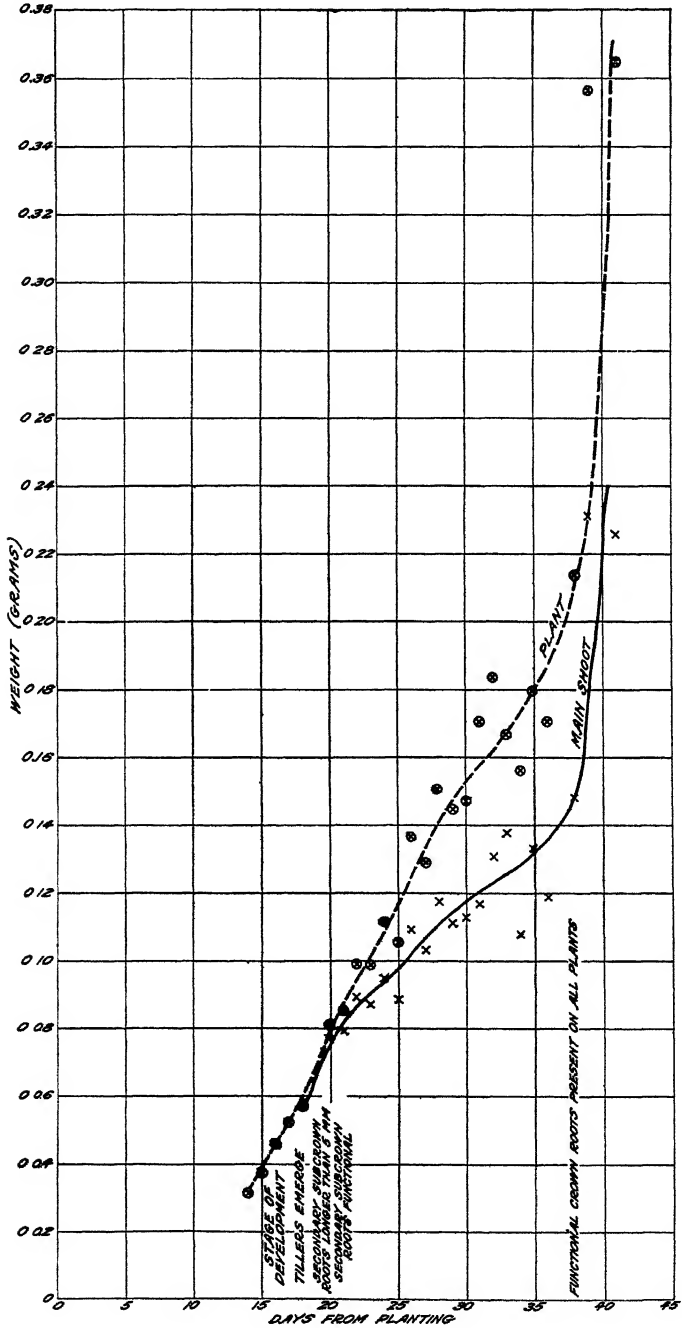


FIGURE 10—Early growth in dry weight of Hannchen barley, greenhouse grown

barley was again determined daily from 10 plant samples grown in the greenhouse at the Arlington farm during the winter of 1930-31 (Fig 10). The ranges and variations in temperature were fairly uniform from day to day and may be disregarded. On the eighteenth day tillers were first large enough to be detached and weighed separately. Two days later secondary subcrown roots had appeared, and slightly exceeded 5 mm in length. These roots became functional the next day. Evidently this accession of root tissue was insufficient to supply nutrients to both main shoot and tillers, for while the growth rate of the plant was maintained almost without change, the rate of main-shoot growth slowed up materially until the thirty-eighth day, when all plants first acquired functional crown roots and their main shoots attained a new maximum growth rate.

Like Tennessee Winter, Hannchen (fig 8) shows no perceptible inflection of the weight-growth curve of the shoot without the kernels for a number of days. The slight inflection on June 15 becomes marked on June 20, about 12 days after flowering.

### DISCUSSION

A number of investigators (1, 10, 11, 15, 16, 18) have shown the existence of correlations between portions of the growth curve and definite stages in the development of the plant. Some (4, 6, 11, 13, 14, 16, 19, 21) believe that the water or nutrient supply is the limiting factor in certain growth stages, or that competition for this material occurs between different parts of the plant.

The data herein presented indicate that lack of sufficient root tissue is a factor that inhibits increase in dry weight in the barley plant before crown roots begin to function. Another flattening of the dry-weight curve, which is even more apparent in the length curve, occurs at the time of rapid tiller development and is accompanied by enhanced growth of crown roots. The development and growth of the spike primordium into the full-sized flowering head is accompanied by a rapid elongation and increase in dry weight of the whole shoot. Up to the beginning of this period of rapid elongation ("jointing" or "shooting") the amount of absorptive tissue evidently has been a factor limiting the growth of the plant. At the time of most rapid tiller growth these young parts, growing much more actively than the main shoot, may compete successfully for the nutrients. The rapid growth of the tillers is accompanied by an increase in root growth until the tillers are nearly the size of the main shoot, when there is sufficient root tissue to supply both. Active jointing then follows. Another factor now enters to complicate the process. In order to maintain an upright posture the jointing shoot must be strong enough not only to support its own weight but successfully to resist wind action and at maturity to carry a heavy load of starch-laden seeds. This condition is met by the deposition of seemingly inactive materials, such as cellulose, lignin, silica, etc., in the culm. This process, of course, rapidly takes much tissue out of the realm of active metabolism, thereby slowing down the rate of growth. There is for some time, however, enough actively growing tissue to more than counterbalance this loss. But barley is a plant of determinate growth. Normally there is no branching at the upper nodes with the exception of the modified branches and leaves which constitute the floral organs in the

curve, as is shown by its regular and steep ascent. As plant parts mature, with the accumulation of inactive substances such as cellulose and lignin, there is relatively less tissue capable of growth. Such inactive tissue is no longer "principal," and "principal" increases more slowly or at a rate directly proportional to the actual amount of actively growing tissue. If a sufficient amount of actively growing tissue is maintained, the growth rate will continue constant. Such a condition exists in plants of indeterminate growth, such as the tomato. Here increase in size continues until the production of organs, such as flowers and fruits, destroys the requisite nutritional balance for active growth of the vegetative growing point. In a plant of determinate growth, such as barley, where branching of the shoot does not occur normally and the spike is produced at the apex, growth continues in the culm and spike until inactive materials are laid down in all parts in sufficient amount to hinder enlargement. Growth will then stop.

In the barley kernels growth limitation is largely due to starch congestion. Such large amounts of this material are stored in the endosperm cells that mitotic division ceases, and under favorable conditions all the available cell space is filled with starch. This has been demonstrated by Cobb (3) in wheat. This deposition of starch in amounts sufficiently large to hinder cell division places a limit on the maximum size of the kernel. Starch deposition proceeds until prevented by drying out, lack of necessary materials, or the tension of the cell walls.

The slackening of top growth during root formation, as noted by Pearsall (16) and Priestley and Evershed (18), probably is due, in part at least, to the inability of available roots to absorb nutrients in sufficient amount to balance the photosynthetic activity possible in the leaves and stem.

Any disappearance of water from the plant at flowering, as reported by Van de Sande-Bakhuyzen (20), would seem to be due merely to deposition of inactive material and consequent drying out of tissues that are approaching senescence. However, Hurd-Karrer and Taylor (12, p. 397) found in their work with wheat that "the physiological processes involved in flowering did not exert any specific effect on their water content."

#### SUMMARY AND CONCLUSIONS

The growth curve of the barley shoot without kernels is typically sigmoid, with certain slight variations from a perfectly smooth curve in both early and late stages. Such a curve is a composite picture of the grand periods of growth of all the organs and also of each individual cell in the measured individual.

Variations in the curve of early growth are associated with definite events in the development of the plant. The hypothesis that the structural inability of the existing roots to absorb nutrients is the factor inhibiting growth in length is supported by the fact that this inhibition is removed as soon as new roots are formed and become functional.

Growth rate in both length and weight is retarded at about the time first tillers are appearing and before their roots are established. When tiller roots become functional the curve rises more sharply.

The curve of leaf length reaches inflection at about first flowering, but the curves of awn-tip length and total length of shoot reach inflec-

spike The flowering head is the terminus of the shoot If this does not proliferate, length growth must cease and the inflection of the curve of awn-tip length will be the inflection of the curve of shoot growth

In contrast to the findings of Van de Sande-Bakhuyzen and Alsberg (21) with wheat, there is in barley no major inflection in total length or in total dry weight at flowering There is, however, an inflection of the leaf-length curve at or shortly after flowering This latter, however, does not affect the height of the plant The growth habits of a plant must be considered in making such generalizations Both maize and wheat extrude their flower heads above the leaf tips before flowering takes place At anthesis the upward growth is about ready to stop It is very different with barley, where flowering generally occurs while the spike is still inclosed in the boot leaf The last internode of the culm bearing the spike is still young and composed of soft growing tissue and continues to elongate until the spike emerges from the sheath and extends, in most varieties, above the end of the distal culm leaf The basal leaves are, for the most part, dead at the time jointing begins, and necrosis proceeds distally, first appearing in the leaf tips and working back through the blade to the leaf sheath The top or boot leaf begins to die at the tip when the kernels are about one-half or two-thirds their mature age, and the awn tips begin to brown a few days later The culm axis or stalk loses its chlorophyll rapidly at this time However, the leaf sheaths protect the stalk from excessive drying for a considerable time and the vessels are still capable of conducting the soil solution to the still actively growing kernels After the chlorophyllous tissue has ceased to function there probably is still translocation of dry matter from the upper part of the stalk and the spike into the kernels even after the shoot has been harvested This certainly is true when the kernels are immature when harvested (9)

If the growth in dry weight is considered, there is found a state of affairs entirely different from growth in length Not only does the inflection of the curve not occur at flowering, but there seems to be no true inflection at all This is due to the fact that the growing seeds present in the spike serve as storage organs for an amount of dry matter which may equal or exceed the total dry matter contained in the remainder of the shoot The deposition of this material begins actively a few days after fertilization of the egg cell and continues for nearly a month, when structural difficulties abruptly halt growth (8)

It seems to the writer that events occurring at the time of root formation, together with the acceptance of the compound-interest principle, may form the basis of a hypothesis applicable to the whole growth curve of plants. *Postulating favorable environmental conditions, rate of growth in the plant is largely, if not entirely, conditioned by the maximum nutritional opportunities in different parts of the plant and the ability of the plant from a physical and chemical standpoint to take advantage of these opportunities.*

According to this hypothesis the aerial portions of the young plant will grow with increasing rapidity according to the compound-interest formula, provided the roots have an absorbing and carrying capacity sufficient to satisfy the nutritional needs of the plant These "maximum nutritional opportunities" are greatest during the middle of the growth period, where the compound-interest formula best fits the

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tion 5 days later in the Tennessee Winter variety and 8 to 10 days later in Hannchen

The curve of shoot weight, excluding kernels, shows inflection 4 days after flowering in Tennessee Winter and 10 to 12 days after flowering in Hannchen.

The total weight of the shoot, including kernels, increases at a regular rate until about the hard-dough stage of the kernel, when the leaves and awns have begun to die

After the hard-dough stage the shoot loses weight on account of mechanical breakage and leaching by rain.

Indirect evidence indicates that growth rate in the plant varies with the accessibility of nutrients and the structural ability of the plant to translocate them and utilize them in growth

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# CATALASE ACTIVITY IN RELATION TO THE GROWTH CURVE IN BARLEY<sup>1</sup>

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## INTRODUCTION

Phenomena associated with growth might be expected to exhibit a grand period paralleling that of growth itself. Catalase activity is well-nigh universal in living, growing tissue. It has been found to be correlated with various phases of metabolism, but its nature and function are problematical. During a study of the growth curve of barley a parallel investigation was made upon catalase activity. The results are reported in this paper.

## REVIEW OF LITERATURE

Weiss and Harvey (14)<sup>3</sup> found catalase activity to be strongly correlated with growth in the proliferation produced by the potato-wart disease in spite of the high acidity of the growing tissues.

Heinicke (8) found not only that there was more catalase activity in the leaves of apple trees fertilized with sodium nitrate than in those of the checks, but also that when only half the plant was given nitrate the portion directly above gave an increased catalase reaction. This indicated that the unfertilized half of the plant could be used as a check. Heinicke suggests that, since catalase activity shows the nitrogen effect in the apple when chemical analysis gives no significant difference in nitrogen content, "it is very probable that the ability to decompose hydrogen peroxide is a more sensitive measure of the metabolic status of the tissue than the usual chemical analysis." He makes an interesting inference when he says.

Many of the preparations of apple-leaf tissue show greater power to decompose hydrogen peroxide than is reported in the literature for tissue from organs more actively engaged in growth processes.

Auchter (2) confirmed the findings of Heinicke on apple-leaf tissue and found that catalase activity was greatly increased in the leaves whenever nitrate of soda was applied to plants of privet, oak, and peach. In all these the growth became more vigorous and the plants contained a significantly greater amount of nitrogen than did the check plants to which no nitrate was added.

Shull and Davis (13) state that in the dimorphic seeds of *Xanthium*, the upper seed, which shows a delayed germination, exhibits constantly less catalase per unit of dry matter than does the lower seed.

Rhine (12) believes that catalase activity is somehow related to the presence of oxygen and oxidation. She found, however, that during

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 355.



nently bathed in mercury. The water temperature is equalized by a motor-driven stirrer of the turbine type. The Bunzel tube is shaken at the rate of approximately 204 complete excursions per minute, and the thermostat is kept constant at  $24.5^{\circ}$ . In starting the shaker motor the switch is thrown one second before the minute, which allows the first mixture to occur approximately on the minute.

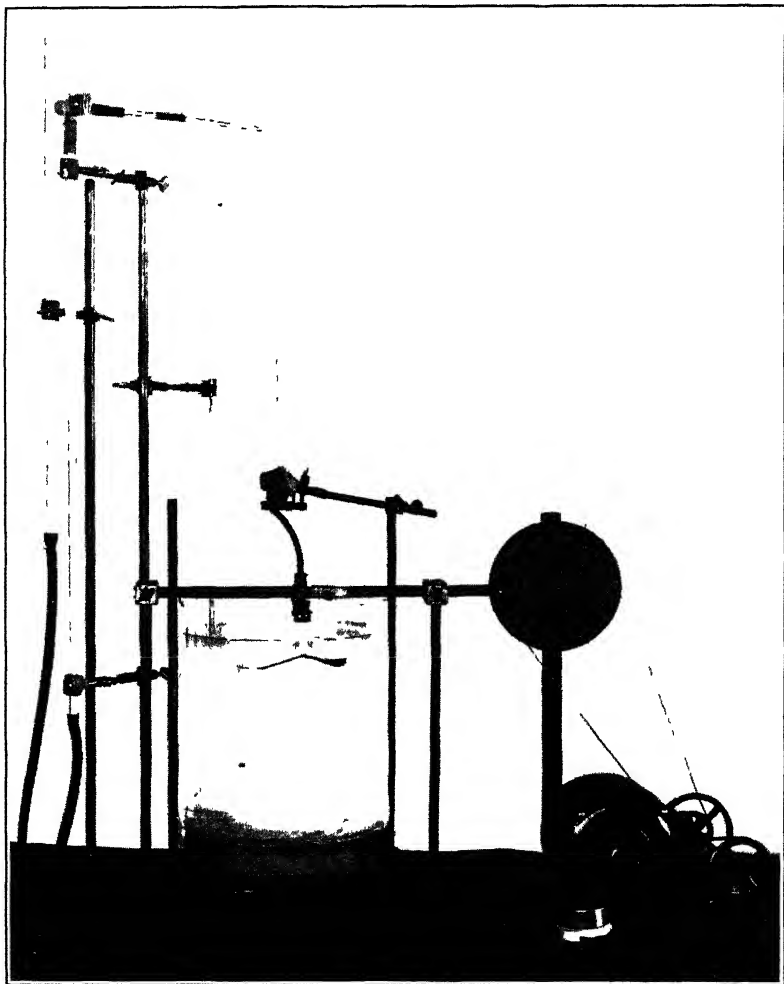


FIGURE 1—Apparatus for the determination of catalase activity (without thermostatic equipment)

Readings are taken at the end of each successive minute for periods of 3, 5, or 10 minutes.

Determinations of catalase activity were made in the following way: The main shoots of five plants of Hannchen spring barley (C. I.<sup>4</sup> No. 531), selected at random from the 20 taken as a single day's

<sup>4</sup> C. I. refers to accession number of Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations

germination as respiration rapidly increased catalase activity decreased, and inferred that the latter can not be a part of the respiratory mechanism. She concludes that catalase activity can be a measure of metabolism only when there is no rapid change in respiration.

Knott (10) found that the youngest and oldest leaves of spinach usually are low in catalase activity, while those intermediate in age have higher and approximately equal activity. His results are open to the criticism that the young leaves probably contained a much lower dry-matter content, which should be corrected for, and the oldest leaves were yellowing and dying.

Ezell and Crist (6) found a significantly negative correlation between catalase activity and average weight of 65 lots of lettuce plants, and Haber (7) found that "catalase activity, growth, and yield were negatively correlated in the vegetatively mature leaves, green-mature fruit, and ripe fruit" in the tomato.

There is much better agreement among plant physiologists than among animal physiologists. Becht's (3) results seem to indicate that the catalase reaction is unreliable as a measure of metabolism in animals. He found a variation of as much as 1,000 per cent in the blood of different dogs under "identical conditions." Morgulis (11) placed frogs under temperature conditions which "it was estimated" caused a change of 300 to 400 per cent in the metabolic rate, and found no accompanying influence on the catalase content of the frogs. He concluded that "it is certain that it [catalase activity] is certainly not a measure of metabolic activity."

Burge (4) and Burge and Burge (5) are convinced that catalase activity is definitely associated with metabolism and with respiration in particular.

#### MATERIAL AND METHODS

Preliminary experiments were run to determine the most nearly practicable optimum conditions for measuring catalase activity in the tissues studied. The methods so determined were used throughout the investigation.

The apparatus used (fig. 1) is a modification of the one described by Appleman (1). It consists of a square, wooden, motor-driven arm sliding through supports at either end and carrying a Bunzel tube (22 mm inside diameter), each arm of which has ample capacity for 4 c. c. of liquid. A flexible rubber tube of sufficient length to allow a full excursion of the shaker arm connects the Bunzel tube with a small-bore glass tube. This glass tube is in direct connection through a 3-way glass stopcock with the upper end of a 50 c. c. burette, the lower end of which is connected by a thick-wall rubber tube with the lower end of a second burette of the same capacity which may be raised or lowered at will to equalize the water levels in the two burettes. The gas-conducting portions of the apparatus are purposely made of small-bore material to reduce the volume of gas subject to temperature and pressure changes. No attempt was made to determine the volume at normal temperature and pressure, because, as noted later, the error from this source is less than that from other variable, uncontrolled factors. The Bunzel tube is immersed in a water thermostat electrically heated and controlled. A knife-type heater is used, and the thermoregulator is of the mercury type, sensitive to about  $\pm 0.02^\circ$  C., working through a mercury relay in which both poles are perma-

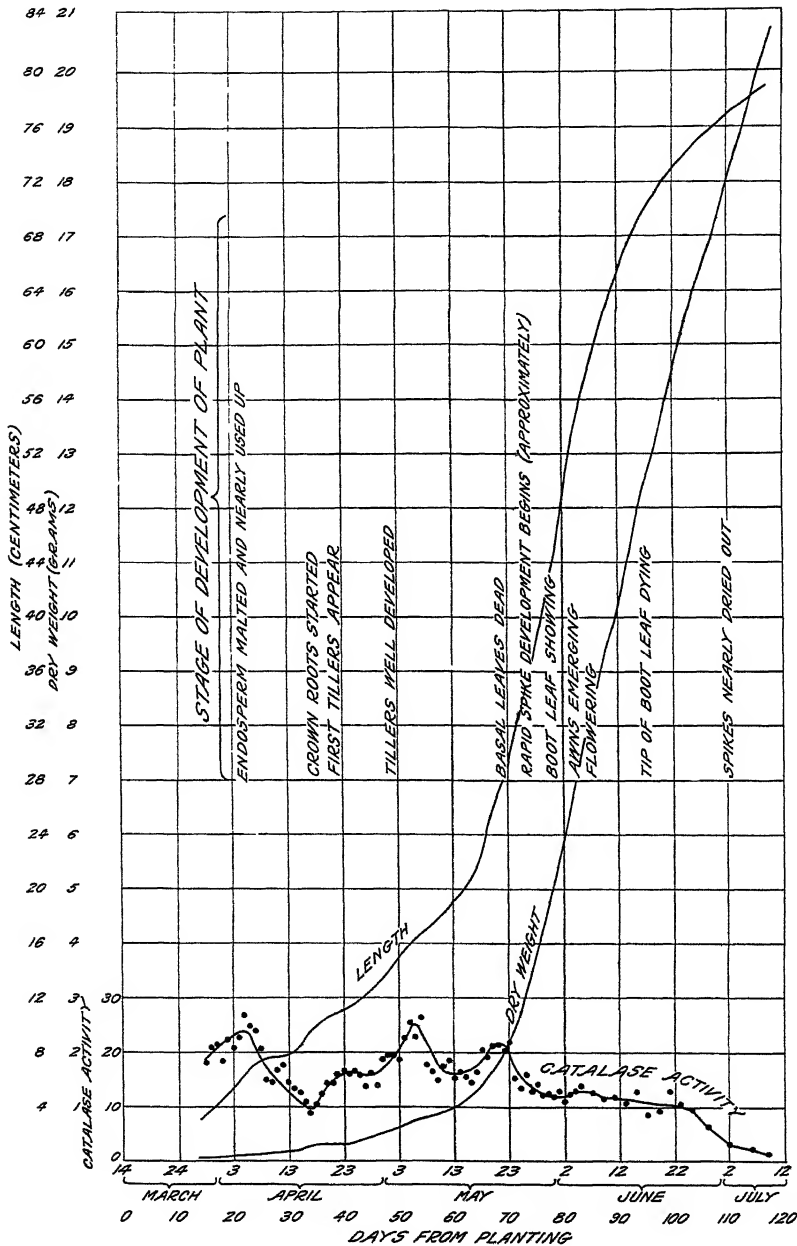


FIGURE 3.—Stage of development, growth in length, dry weight, and catalase activity per unit of dry weight of the main shoot of Haunchen barley from emergence to maturity, grown in plots at the Arlington Experiment Farm, Rosslyn, Va., 1928

sample for growth study, were clipped finely with shears and mixed thoroughly. From this composite a 5-g sample<sup>5</sup> was weighed out and quickly treated with powdered calcium carbonate, the cut ends being covered to prevent acidity from developing at those points. A small quantity of water and where necessary a little quartz sand that had been carefully cleaned and thoroughly washed were added and the mixture was reduced in the mortar to a thin paste. Distilled water<sup>5</sup> was then added to make 250 cm<sup>3</sup>. The mixture was thoroughly stirred, and a 2 cm<sup>3</sup> sample, which contained 0.04 g of green tissue, was pipetted into one arm of a Bunzel tube. In the other arm of the tube was placed 2 cm<sup>3</sup> of 12-volume Dioxogen (hydrogen peroxide), and the tube was attached to the shaker cork in the thermostat and left three minutes to come to temperature equilibrium. The 3-way

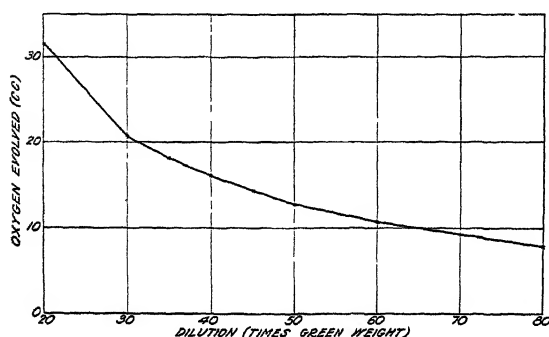


FIGURE 2—Relation between the volume of oxygen liberated from hydrogen peroxide by 2 cm<sup>3</sup> of a tissue suspension and the dilution of that suspension

cock in the top of the burette was opened and water leveled to 0.0 cm<sup>3</sup>. At the end of the 3-minute period the cock was turned so that passage from the Bunzel tube was open to the burette. One second before the minute the switch was thrown, and the shaking began very nearly on the minute. Gas immediately began to be evolved on the mixture of the hydrogen peroxide and the diluted plant material, and the displaced gas was collected in the burette. The water surfaces in the burette and the leveling tube were leveled off and the number of cubic centimeters of gas evolved was read, estimating to 0.01 cm<sup>3</sup>, at the end of each minute for 10 successive minutes. Duplicate determinations were made. If the total amounts differed by as much as 1 cm<sup>3</sup>, a third sample was run and the average of the three used. The average volume of gas evolved in 10 minutes was calculated for 0.008 g of dry matter, and this figure was taken as the "catalase activity" of the sample. This seems valid, as preliminary experiments have shown that through a rather wide range of dilutions catalase activity is proportional to the dilution (Fig. 2.)

Dry matter determinations were made upon duplicate 5-gm samples dried to constant weight at 80° C in a vacuum of approximately 28 inches of mercury. After flowering, when the kernels in the spike began to grow rapidly, the shoot was divided into spike and culm and dried in separate samples. The percentage of dry matter for total shoots was figured from the two determinations.

Throughout the investigation care was taken to give all samples as nearly identical treatment as possible, and the results are believed to be consistent.

<sup>5</sup> g and cm<sup>3</sup> are the abbreviations recently adopted by the Government Printing Office for grams and cubic centimeter, respectively

TABLE 1 — *Average length, average dry weight, and catalase activity in the main shoot of spring-sown Hannchen barley, 1928—Continued*

Day of harvest		Length	Dry Weight	Catalase activity (O <sub>2</sub> evolved in 10 minutes)	
Date	Days after planting			Per 0.04 gm green tissue	Per 0.008 gm dry matter
		<i>Cm</i>	<i>Grams</i>	<i>Cm<sup>3</sup></i>	<i>Cm<sup>3</sup></i>
May 25.....	72	32 73	3434	12 54	13 38
May 26.....	73	31 57	2455	14 27	15 72
May 27.....	74	36 01	3323	12 58	12 80
May 28.....	75	34 59	2911	13 52	14 00
May 29.....	76	39 88	3948	12 54	12 12
May 30.....	77	42 51	4333	11 94	12 37
May 31.....	78	44 21	5081	13 74	11 93
June 1.....	79	45 18	5023	14 39	12 70
June 2.....	80	52 53	7169	12 62	10 89
June 3.....	81	53 35	6965	14 03	12 18
June 4.....	82	51 89	6535	13 24	12 98
June 5.....	83	56 50	6786	15 78	13 84
June 7.....	85	58 08	8311	14.58	12 59
June 9.....	87	59 83	8287	14 99	11 58
June 11.....	89	63 66	1 0519	16 38	11 88
June 13.....	91	64 00	1 0030	18 07	10 67
June 15.....	93	65 48	1 2159	17 86	12 93
June 17.....	95	68 56	1 3391	12 78	8 57
June 19.....	97	62 15	1 1565	13 00	9 18
June 21.....	99	66 99	1 4706	18 10	12 93
June 23.....	101	64 70	1 4705	16 89	10 43
June 25.....	103	64 89	1 4973	17 11	9 66
June 28.....	106	70 15	1 9455	17 93	6 34
July 2.....	110	68 50	1.6613	7 23	3 10
July 6.....	114	69 80	2 0970	5 76	2 24
July 9.....	117	71 47	2 0373	5 09	1 43

While there was a rather wide variation in catalase activity per unit (0.008 g) of dry weight from day to day in the 1928 data, there was a distinct trend apparent, as is indicated by the smoothed curve drawn through the points. There are four clearly defined elevations in the first 70 days of growth. During this time the plant was composed mainly of succulent actively growing tissue. The dry matter did not reach 20 per cent until the seventy-sixth day. After the seventieth day (May 23) the percentage of dry matter increased, gradually at first and then very rapidly as the seeds dried out. On May 23 the basal leaves were dead. The culm leaves soon died also, and on June 17 the boot leaf was dying, the maximum moisture remaining longest in the nodes and kernels. From May 23 the catalase activity decreased quite uniformly until June 25, when final drying out occurred and the catalase activity dropped to 1.43 cm<sup>3</sup> at the final determination. This drop in catalase activity is associated with rapid growth beginning about the seventieth day, the relation continuing unchanged up to the point where growth abruptly ceases.

Catalase determinations were run upon one planting only in 1928. To verify these data and to check their apparent relation to stage of plant growth, three supplementary series of determinations were run subsequently upon Hannchen barley. None of these latter, however, carried the plant to maturity. Only the early stages, where the earlier curve was more variable and consequently more subject to doubt, were studied in these later experiments. Figures 4, 5, and 6 show graphically the shoot length, dry weight of shoot, and catalase activity corrected for dry matter, together with the appearance of certain developmental stages in each of the three series. The first series was run upon material grown in the greenhouse in the late winter and spring of 1929.

## CURVE OF CATALASE ACTIVITY IN THE BARLEY PLANT

In Table 1 are shown the following data for the main shoot of barley: Average length in centimeters, average dry weight in grams, catalase activity found in 0.04 g of green tissue, and the catalase activity calculated for 0.008 g of dry matter of the daily samples

In Figure 3 the amount of catalase activity per unit of dry weight in the shoot of the Hannchen barley plant is plotted against time. On the same sheet are shown the curves of total length and total dry weight of main shoot. The stages of development of the plant on different dates also are indicated.

TABLE 1—Average length, average dry weight, and catalase activity in the main shoot of spring-sown Hannchen barley, 1928

Day of harvest		Length	Dry Weight	Catalase activity (O <sub>2</sub> evolved in 10 minutes)	
Date	Days after planting			Per 0.04 gm green tissue	Per 0.008 gm dry matter
				Cm <sup>3</sup>	Cm <sup>3</sup>
Mar 28.....	14	3.04	0.0051	17.93	17.99
Mar 29.....	15	3.29	0.0056	20.87	20.84
Mar 30.....	16	3.84	0.0058	21.51	21.03
Mar 31.....	17	4.70	0.0073	17.91	18.08
Apr 1.....	18	4.41	0.0072	20.54	22.02
Apr 2.....	19	4.58	0.0086	24.37	20.74
Apr 3.....	20	5.51	0.0081	23.51	22.42
Apr 4.....	21	5.76	0.101	30.64	26.71
Apr 5.....	22	6.19	0.106	25.46	24.61
Apr 6.....	23	7.65	0.128	28.64	23.84
Apr 7.....	24	6.76	0.150	19.21	20.06
Apr 8.....	25	7.43	0.143	15.91	14.98
Apr 9.....	26	7.56	0.162	13.29	14.22
Apr 10.....	27	7.07	0.164	17.28	16.54
Apr 11.....	28	7.53	0.173	17.26	17.42
Apr 12.....	29	8.11	0.164	14.33	14.33
Apr 13.....	30	7.35	0.187	13.32	13.43
Apr 14.....	31	8.14	0.184	12.92	12.67
Apr 15.....	32	8.25	0.200	11.32	10.95
Apr 16.....	33	9.08	0.245	10.10	8.72
Apr 17.....	34	9.77	0.288	10.73	10.21
Apr 18.....	35	9.25	0.262	13.40	12.05
Apr 19.....	36	10.36	0.110	12.20	14.24
Apr 20.....	37	11.16	0.290	15.05	14.03
Apr 21.....	38	10.80	0.334	13.35	15.93
Apr 22.....	39	11.23	0.284	13.29	16.40
Apr 23.....	40	10.96	0.289	14.18	15.73
Apr 24.....	41	11.00	0.342	14.50	16.30
Apr 25.....	42	12.15	0.408	13.40	15.64
Apr 26.....	43	12.77	0.437	11.25	13.88
Apr 27.....	44	11.07	0.329	14.06	16.00
Apr 28.....	45	12.19	0.406	13.95	13.90
Apr 29.....	46	13.06	0.505	17.07	18.45
Apr 30.....	47	13.07	0.535	16.82	19.31
May 1.....	48	14.09	0.531	16.89	19.14
May 2.....	49	14.16	0.568	16.72	18.61
May 3.....	50	15.70	0.606	17.74	22.48
May 4.....	51	17.29	0.751	21.80	25.29
May 5.....	52	16.11	0.692	18.12	22.76
May 6.....	53	16.68	0.779	22.63	26.34
May 7.....	54	17.84	0.873	16.47	17.45
May 8.....	55	16.98	0.809	13.50	16.20
May 9.....	56	16.47	0.731	13.93	14.91
May 10.....	57	19.15	1.024	15.50	17.16
May 11.....	58	18.33	0.927	16.65	18.14
May 12.....	59	17.09	0.792	14.75	15.28
May 13.....	60	17.91	0.950	15.64	16.21
May 14.....	61	20.39	1.224	14.23	15.46
May 15.....	62	20.24	1.075	14.16	14.35
May 16.....	63	19.56	1.123	15.73	16.08
May 17.....	64	21.77	1.373	18.65	20.33
May 18.....	65	23.94	1.533	17.15	19.19
May 19.....	66	23.31	1.453	18.91	21.09
May 20.....	67	25.55	1.516	17.96	21.20
May 21.....	68	28.04	1.781	17.57	20.21
May 22.....	69	29.79	2.258	18.24	21.79
May 23.....	70	31.92	2.387	14.93	15.13
May 24.....	71	31.70	2.789		

On account of environmental differences a definite stage of development naturally did not occur at the same age in days in all four experiments. In order to compare properly the curves in Figures 3, 4, 5, and 6, the data shown therein are plotted against the six definite developmental stages indicated

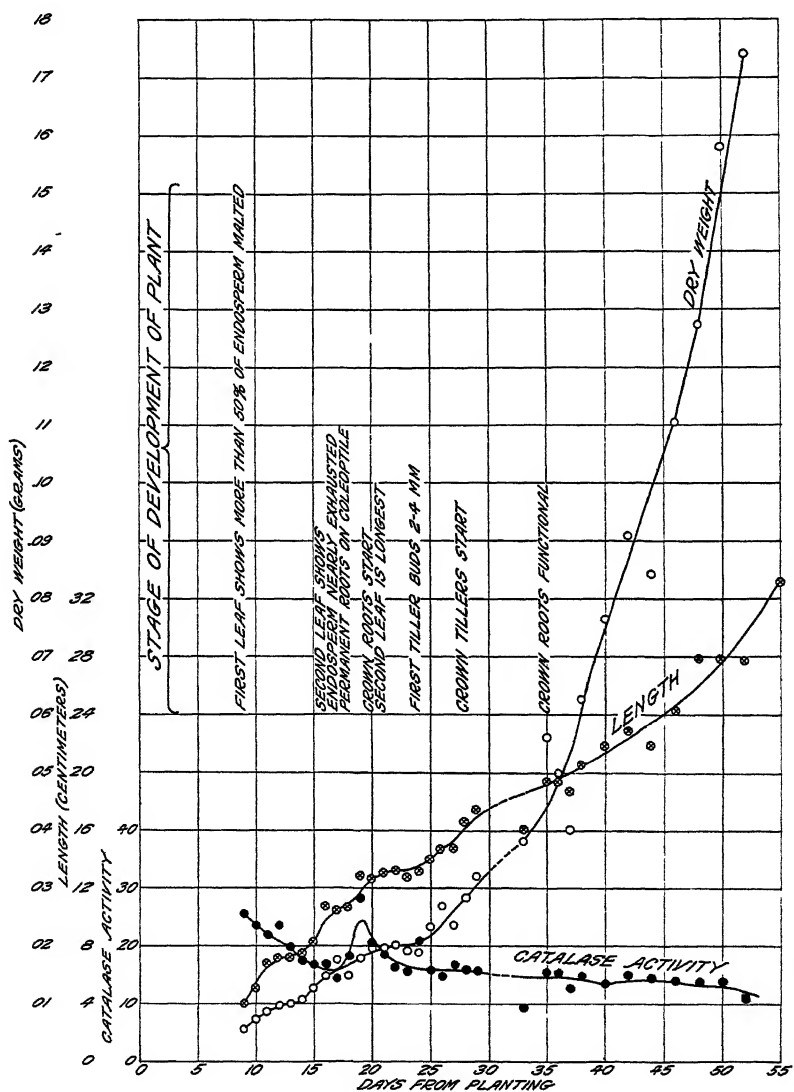


FIGURE 5—Stage of development, growth in length, dry weight, and catalase activity per unit of dry weight of the main shoot of Hamchurch barley grown in plots at the Arlington Experiment Farm, Rosslyn, Va., 1929

The resulting curves (fig 7) are the ones already shown, lengthened, or shortened to fit the developmental stages indicated on the abscissa of each original curve. This makes possible a direct approximate comparison of the four series of determinations.

This material was limited, and catalase determinations were not continued much beyond the time when the crown roots became functional. The curve in Figure 4 corresponds fairly well with that for the data previously obtained, except that the first low point does not occur so near the time of endosperm exhaustion as in the former.

The second series was run upon material grown in field plots in 1929. The curve in Figure 5 almost parallels that of the greenhouse material of the same year. On account of poor soil conditions the plants did not tiller well and appeared so abnormal that the experiment

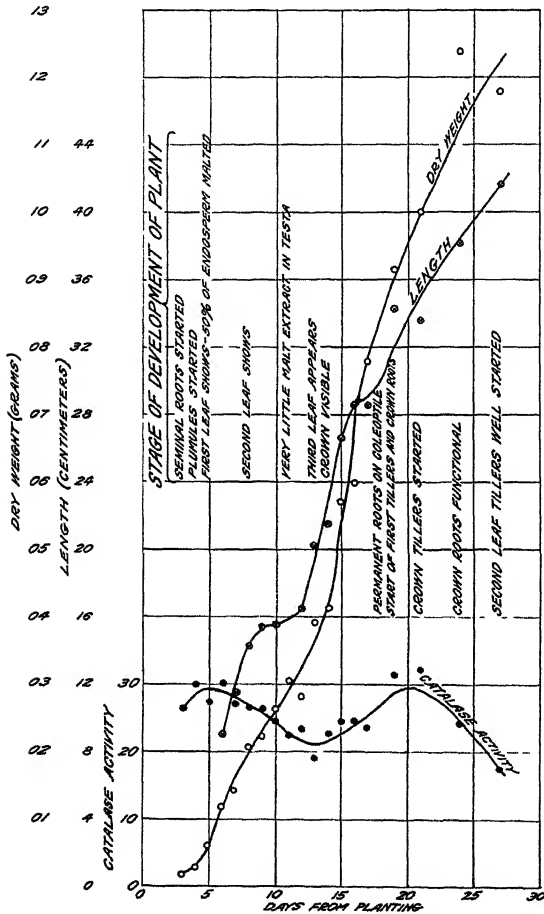


FIGURE 4.—Stage of development, growth in length, dry weight, and catalase activity per unit of dry weight of the main shoot of Hamelien barley grown in the greenhouse at the Albington Experiment Farm, Rosslyn, Va., in the winter of 1928-29

was discontinued on the fifty-second day. In the graph it is seen that from days 9 to 16 growth was rapid while the curve of catalase activity dropped. From days 17 to 24 growth lagged and an elevation in the catalase curve occurred. After day 24 rapid growth rate was again accompanied by a decreased catalase activity.

The third series was run upon material grown in the greenhouse in the winter of 1929-30. Catalase determinations were made until jointing was well under way. In this series each determination was made upon the whole of the main shoot of a single plant, except in the early

stages, when more material was desirable. The resulting curve (fig 6) is reasonably smooth and resembles the other three quite closely. There is, however, no elevation during the time of active tiller production, as there is in the 1928 curve. The increase during jointing and immediately preceding the appearance of the floral organs, however, is significant and agrees with the 1928 data. Here also growth rate is rapid while catalase activity drops off. When growth lags the curve of catalase activity rises.

## DISCUSSION

The only measure of catalase activity is the volume of molecular oxygen liberated when the substance under investigation is brought into intimate contact with hydrogen peroxide. The presence of hydrogen peroxide in living tissue has not been proved. Consequently no assumptions can be made as to the nature of the reaction, its function, or its usefulness to the plant. Furthermore, if catalase activity be destroyed in normal tissue, as happens in determinations in the laboratory, no idea can be had of the total amount produced in the plant, the determinations then show merely the excess of manufacture over destruction.

In this study of catalase activity a composite sample of all the tissues of the entire main shoot was used. This was composed mainly of meristematic and young, actively growing tissues in the seedling stage. As age increased, the leaves rapidly matured, the stem was differentiated and became woody, and finally all the tissues except the seeds in the spike were in a dying condition. This course of events produces the typical sigmoid curve of growth. Had meristematic tissue alone been used for the daily catalase determinations, the curve

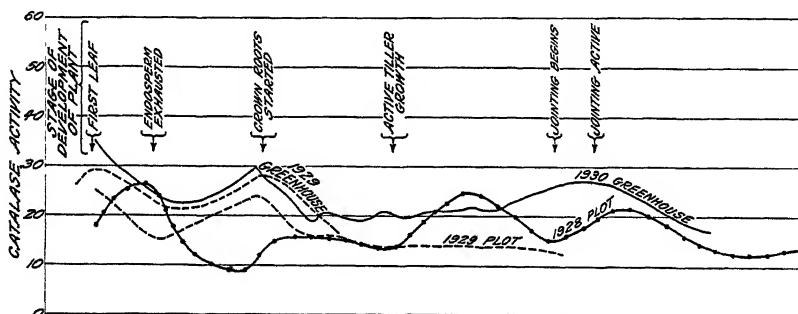


FIGURE 7—Four series of catalase activity determinations plotted against stage of development (See figs. 3 to 6, inclusive)

probably would have differed as much from the one shown herein as a growth curve of meristematic tissue would differ from one constructed from entire-shoot data.

A low C/N ratio generally is accepted as necessary to active metabolism. Auchter's data (2) on woody plants indicate that an increase in carbohydrates in proportion to the nitrogen present is accompanied by a decreased catalase activity. Hicks (9) found in wheat the lowest C/N ratio in the distal end of an organ, for instance, the leaf tips have a lower C/N ratio than the bases even though the meristem of the leaves is at the base. From these results one would expect to find the greatest catalase activity in barley in tissues that are mature but not dying, and in plants that have a relatively large amount of mature living tissue. In the writer's determination of catalase activity in different parts of the barley plant<sup>6</sup> the mature portions seemed to have a higher catalase activity than did the very young tissues or those much past maturity, for instance, the flag of the boot leaf contained an amount greater than that of any other part tested, and the sheath of

<sup>6</sup> Unpublished data.

Considering the 1928 data alone, it seems possible to correlate increased intensity in the curve of catalase activity with the plant stages characterized by active metabolism. The data from the three subsequent series of determinations, however, indicate that so definite and inclusive a conclusion is not entirely justified. In all cases in 1928 (fig 7) intensity of catalase activity is high in the germinating plant and gradually falls off during the rapid growth preceding the exhaustion of the endosperm. There then occurs a general slackening of

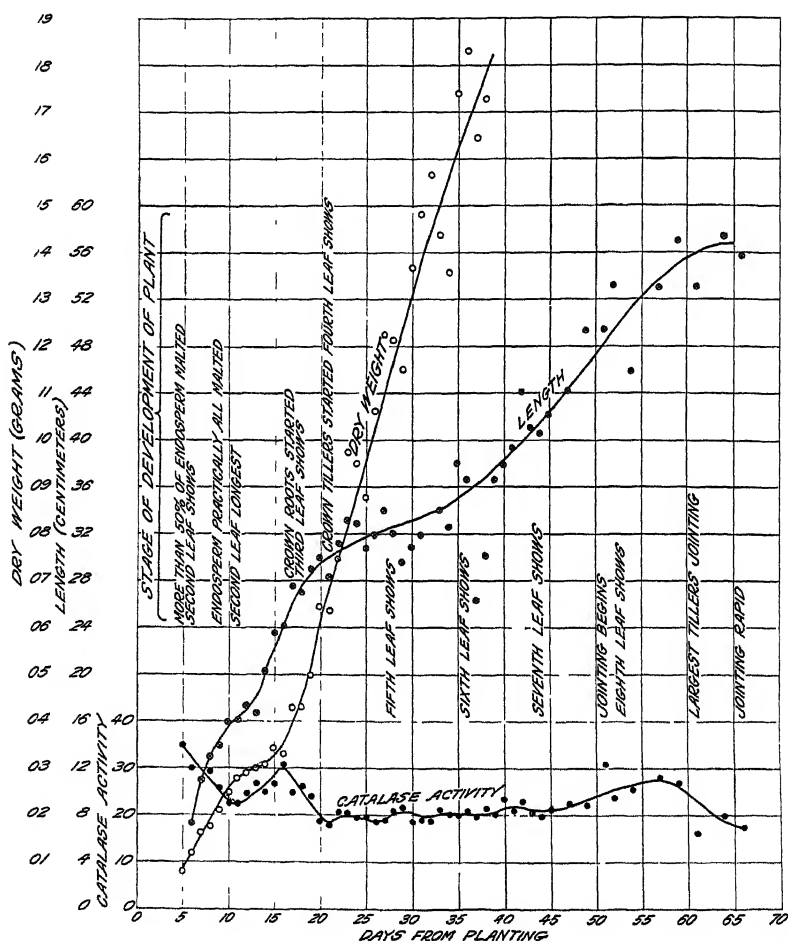


FIGURE 6.—Stage of development, growth in length, dry weight, and catalase activity per unit of dry weight of the main shoot of Hannechen barley grown in the greenhouse at the Arlington Experiment Farm, Rosslyn, Va., in the winter of 1929-30

growth rate, as indicated by dry-weight determinations. At the same time catalase activity per unit of dry matter begins to climb until the crown roots are functional, after which growth again becomes rapid and catalase activity decreases in intensity. There is evidence of another period of high catalase activity during jointing and just prior to the first appearance of the spike, with some indications of a high during the previous period of tiller production. As senility proceeds in the plant, catalase activity decreases and drops to a low level as the vegetative part of the plant dies.

## SUMMARY AND CONCLUSIONS

Four series of daily determinations of catalase activity were made during the growth period of Hannchen barley in field plot and greenhouse. The first series was carried through to the mature plant; with the other three series the studies were continued through the period of early growth, where the results were most variable.

Catalase activity (1 e, cubic centimeters of oxygen gas liberated per unit of dry weight) is roughly proportional to the reciprocal of growth rate. Three hypothetical explanations are suggested.

The curve of catalase activity shows three peaks, each occurring at the time of inception of a new and definite stage in the functional activity of the plant.

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the boot leaf held second place in one variety and third in another, while the awns held third and second place, respectively. All the parts of the shoot, including the kernels, proximal to the awns were significantly lower in catalase activity.

It is evident that the total catalase activity in the plant approximately keeps pace with growth. But in order to make catalase exactly proportional to growth the curve of catalase activity (expressed as cubic centimeters of oxygen evolved per unit of dry matter) must be a straight line parallel with the horizontal axis. It will be seen that this condition does not obtain. There are certain elevations above the mean of the curve which must be explained. In all four series of determinations oxygen evolution is high at and immediately following germination, but drops off with active seedling growth and reaches a low point shortly after all the endosperm is malted. Growth then slackens somewhat, and catalase activity again climbs, reaching a second peak at the time of the appearance of the crown roots. Thereafter tiller buds appear and growth is accelerated in the plant and the curve drops to a level, which, in the 1930 greenhouse experiments, is maintained with but slight deviation until the peak during early jointing. In the 1928 plot material this peak during early jointing is preceded by another high point during active tiller growth, which does not appear in any other curve. During late jointing the spikes develop rapidly and catalase activity drops off (fig 3) to a level which is maintained fairly constant until the actively growing portions of the plant are proportionately reduced by the deposition of a considerable amount of inactive structural material and the progressive death of the leaves from crown to spike.

From the foregoing it appears that the determinations of catalase activity are roughly proportional to the reciprocal of growth rate, being, in general, lowest during the stages of most active growth, as measured by length and by deposition of dry matter. Furthermore, three very definite elevations in the curve are evident: (1) During early germination; (2) during the development of the crown roots and before they become functional, and (3) during early jointing, which immediately precedes the appearance in the boot leaf of the floral spike. In other words, each peak in the curve occurs at the inception of a new and definite stage in the functional activity of the plant.

It is evident that the substance involved in catalase activity is produced during growth and that this production is, as has been intimated, approximately proportional to the amount of living tissue producing it. It is possible that the peaks in the curves may be explained by one of a number of assumptions. In the first place, it may be that the substance is produced at a uniform rate by the living cell, but is used up in the process of growth, and is more rapidly used up in rapid growth. In the second place, an old cell may be a larger producer than a young cell; consequently, during rapid active growth, when a proportionately large number of the cells are young, the average production would be lowered and the lagging production would result in a drop in the curve. In the third place, it might be assumed that relatively great physiological activity actually precedes rapid extension in length and deposition of dry matter.

# FACTORS INFLUENCING THE BLOOD-SUGAR LEVEL OF DAIRY CATTLE<sup>1</sup>

By R E HODGSON, formerly *Graduate Assistant*, W H RIDDELL, *Dairy Production Investigator*, and J S HUGHES, *Animal Nutritionist*, Kansas Agricultural Experiment Station

## INTRODUCTION

The sugar of the blood of dairy animals has been the subject of considerable investigation in its relation to milk secretion and disease. It is the precursor of the lactose of milk. Meigs (11)<sup>2</sup> has presented a thorough review of the literature on this little-known phase of metabolism. There is good evidence to show that milk sugar is derived from the glucose of the blood, though the process by which the transformation takes place in the mammary gland remains obscure.

In recent years the behavior of the blood sugar has been studied in dairy cattle under certain abnormal conditions. Several investigators have presented figures on the normal concentration of this constituent. Hayden and Sholl (5) after 75 tests with 44 cows concluded that the average concentration of sugar in the blood of dairy cows is 51.75 milligrams per 100 cubic centimeters. Moussu and Moussu (9), working with only 10 cows of different breeds, gave a range of 0.061 to 0.08 per cent, while Hayden and Fish (4), as a result of 68 analyses, gave an average figure of 46.52 milligrams per 100 cubic centimeters with a range of 30 to 70 milligrams. In a later and more extensive piece of work, Hayden (3) reported the low average of 41.15 milligrams of sugar per 100 cubic centimeters of blood for 253 samples taken from 23 cows over a period of 11 months.

Scheicher (12), in studying the ratio of blood sugar to lactose in dairy cattle, observed that the sugar concentration of the blood ranged between 0.055 and 0.10 per cent and averaged 0.0744 per cent, while Anderson and his associates (1) in a recent investigation gave a range of 43.2 to 142.0 milligrams for animals of all ages and an average of 51.2 milligrams for animals in the older group.

It is evident from these results that the blood-sugar level of dairy animals, while tending toward a mean value somewhere near 50 milligrams per 100 cubic centimeters of blood, is subject to wide variations. The causes of these variations have not been discussed to any extent in articles reporting investigations in this field. Undoubtedly, however, they are due, in part, to the fact that the rate at which sugar enters the blood and the rate at which it leaves the blood vary under different conditions. The actual amount of sugar in the blood at any time depends on the relative intensity of these two opposing sets of conditions.

Figuratively speaking, there are three streams of sugar supplying the blood. One enters from the intestines, another arises from the hydrolysis of glycogen, and a third from the synthesis of sugar from

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<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 364.



## FACTORS STUDIED

## AGE

The data collected comprise 20 observations made on not less than eight animals in each of 11 age intervals. Animals of the four major dairry breeds were used. The mean blood-sugar values were calculated, and the results are assembled in Table 1.

TABLE 1—*Influence of age on the blood sugar<sup>1</sup> of dairry cattle*

Age class of animals (inclusive)	Mean value for blood sugar <sup>2</sup>	Age class of animals (inclusive)	Mean value for blood sugar <sup>2</sup>
	<i>Mg per 100 cm<sup>3</sup></i>		<i>Mg per 100 cm<sup>3</sup></i>
1 to 6 days.....	100.4±1.685	16 to 19 months.....	62.2±1.000
1 to 4 weeks.....	88.2±1.226	20 to 23 months.....	55.0±.685
1 to 3 months.....	80.2±1.212	24 to 47 months.....	54.6±.770
4 to 7 months.....	75.4±.794	48 to 71 months.....	53.6±.795
8 to 11 months.....	69.6±1.123	72 to 96 months.....	53.4±.669
12 to 15 months.....	67.8±.821		

<sup>1</sup> 20 determinations of blood sugar on not less than eight animals were averaged for each age interval.

<sup>2</sup> gm and cm<sup>3</sup> are the abbreviations recently adopted by the Government Printing Office for milligrams and cubic centimeters, respectively.

It is apparent that during the early stages of life there is a close inverse relationship between the blood-sugar content and the age of dairry cattle. A mean of 100.4±1.685 milligrams sugar per 100 cubic centimeters of blood was obtained in this study for calves less than 1 week of age. As the animal grows older its blood-sugar concentration decreases until it averages approximately 54 milligrams, when the animal is 2 years old. After this age is reached, the blood-sugar level does not seem to be influenced to any appreciable degree by an increase in age.

Two hundred and twenty-two observations made during the winter months on 74 animals between 2 and 8 years of age gave a mean blood-sugar concentration of 53.03±0.297 milligrams per 100 cubic centimeters of blood, with values ranging from 35 to 74 milligrams.

## BREED

The mature cows in the herd were used for the study of the influence of breed on blood-sugar content. By using only mature animals it was sought to exclude the age factor. Stage of lactation was not considered since these studies have shown it to have little influence on the blood-sugar concentration.

No significant difference was observed in the blood-sugar content of the various breeds. An average of 44 determinations on each of the four major breeds gave the following means: Ayrshire, 53.1±0.856, Guernsey, 53.6±0.711, Holstein, 52.8±0.440, and Jersey, 52.5±0.459.

## MILK YIELD

Hewitt (7) has recently reported results with dry and lactating cows. Eight determinations on three dry cows gave an average of 90.4 milligrams blood sugar, while a similar number on three lactating cows gave an average of 50.7 milligrams. From this he concluded that the blood-sugar level in dry cows is decidedly higher than for cows in milk. Theoretically, the withdrawal of sugar from the blood

other compounds. The first of these can be controlled to a considerable extent by limiting the amount and kind of carbohydrates in the feed. In the case of dairy animals where the digestion is slow and more or less continuous, the rate of absorption of monosaccharides from the digestive tract under ordinary conditions probably does not vary sufficiently to produce any marked fluctuation in the blood-sugar level. The second stream, namely, that arising from the hydrolysis of glycogen, is largely under the control of the nervous system. When an animal is excited, the rate of conversion of glycogen into glucose is increased through the action of the adrenal glands. Excitement may produce a pronounced increase in the sugar level of the blood in a short time. The third source of sugar, namely, the synthesis of sugar from other compounds, is controlled by the processes of metabolism. This source of sugar probably produces very little fluctuation in the blood-sugar level under ordinary conditions.

Sugar is being steadily withdrawn from the blood and oxidized to furnish energy for maintaining the various functions of the body. The amount varies greatly, depending on the degree of muscular activity. Sugar is also removed from the blood to provide the lactose in the milk of lactating animals. Under normal conditions any sugar in excess of that needed for the production of energy and lactose is stored as glycogen or converted into fat, in which form it is stored as reserve energy.

A knowledge of sugar metabolism and the factors that affect the blood-sugar level is important in studying the problem of milk secretion. It was in part for the purpose of securing such information that the experiments described in this paper were undertaken.

#### EXPERIMENTAL METHODS

One hundred and forty animals were used in these studies. All belonged to the college herd and were maintained under normal conditions of herd management. They were fed at 6 a. m. and 4 p. m. each day, and milked at 5 a. m. and 4 p. m.

The animals were selected and handled in such a way that information could be obtained concerning the influence of the following factors on the blood-sugar concentration: Age, breed, lactation, fasting, introduction of relatively large amounts of glucose into the stomach, and excitement. In many cases a single determination was used in more than one comparison. In studying the influence of any one factor, care was taken to see that other conditions affecting the blood-sugar level were maintained as nearly constant as possible.

In recent investigations (6, 10) it has been shown that the various methods now in use for the determination of blood sugar may yield somewhat different results. Folin's new micro method (2), which appears to give results in close agreement with the actual sugar content, and which requires only 0.1 cubic centimeter of blood for each determination, was used in this work. The blood was drawn from the ear by means of a capillary pipette and rinsed into a 10 per cent sodium tungstate solution. In all cases the samples were centrifuged and prepared for analysis within one hour after the blood was drawn. The majority of the samples were collected at approximately 9 a. m.

The results, which are summarized in Table 3, indicate that no significant difference in the blood-sugar level of the dairy cow is produced by feeding. This is undoubtedly due to the rather slow and continuous process of digestion resulting from the complex nature of the bovine stomach.

It will be observed from Table 3 that there is a small increase at 3 p. m., which probably can be accounted for by the increased activities about the barn at this time in preparation for the afternoon feeding.

That the rate of absorption of sugar from the digestive tract does influence the blood-sugar level in dairy cows is indicated by the results of the two following experiments—the first on the influence of fasting, and the second on the sugar tolerance of the dairy cow.

#### FASTING

Five dairy heifers were used in studying the influence of inanition on the blood-sugar content. These heifers ranged in age from 1½ to 2 years and comprised 2 Holsteins, 1 Ayrshire, 1 Guernsey, and 1 Jersey. Throughout the trial they were kept in a paddock with shed adjoining. Water was available at all times.

Samples of blood for analysis were drawn daily, beginning two days before the fasting period, and the live weights of the animals determined. At the conclusion of the 9-day fasting period the heifers were gradually returned to normal feeding conditions.

Figure 1 shows the results in graphic form. It will be observed that as the fasting period advanced the concentration of the blood sugar decreased. This decrease was continuous and uniform until the morning of the seventh day, when a substantial increase occurred. This increase may be explained by the fact that the heifers broke through the fence and obtained some roughage the evening before. As the fasting continued the blood sugar decreased more than 50 per cent of its initial content. The lowest average value observed was 28.5 milligrams per 100 cubic centimeters blood for the eighth day of the trial, as compared with an average initial content of 61.2 milligrams.

After nine days without feed the heifers appeared gaunt and inactive. Their feces were watery and contained mucous material, though it is unlikely that all of the contents had been removed from the digestive tract in the short time involved.

The blood-sugar content of the heifers did not return to normal immediately after they were fed, but increased rather slowly for several days. The average live weight of these heifers decreased 120 pounds.

#### ADMINISTRATION OF GLUCOSE

In order to secure more rapid absorption of carbohydrate from the digestive tract, trials were run in which sugar was dissolved and given by means of a stomach pump. The animals in the various tests were fed different amounts of glucose according to their size and capacity. The glucose used was dissolved in water at body temperature and introduced slowly by means of a stomach pump. That much of the solution found its way immediately into the abomasum and was readily available for absorption is indicated by the prompt rise in blood sugar that occurred in a majority of the animals under observation.

to form the lactose of milk might lower the blood-sugar level in lactating cows. It does not seem probable, however, that this factor alone would explain the wide difference observed.

In order to study the influence of milk yield on blood-sugar level, all the available data in these studies were grouped according to daily milk yield, as shown in Table 2. The results on heifers were not grouped with those on dry cows, since, as already shown, young animals have a higher blood-sugar level than mature animals. The difference in the means of the dry and the heaviest producing group ( $1.9 \pm 1.16$ ) is less than twice its probable error, which is not generally regarded as significant. However, a small difference is apparent in the figures presented, and a slight negative correlation ( $0.190 \pm 0.038$ ) was obtained in correlating blood sugar and milk production for the three producing groups in Table 2.

TABLE 2—*Influence of milk yield on blood-sugar level in dairy cattle*

Daily milk yield	Determinations	Cows	Mean value for blood sugar
	Number	Number	Mg per 100 cm <sup>3</sup>
Dry.....	42	20	52.6 $\pm$ 0.843
1 to 15 pounds.....	27	21	52.9 $\pm$ 455
16 to 35 pounds.....	64	33	51.6 $\pm$ 514
Over 35 pounds.....	32	15	50.7 $\pm$ 799

While these results are lower than those reported by Schlotthauer (13), in general, they confirm his finding that heavy producing cows have a slightly lower blood-sugar level.

#### FEEDING

It is a well-recognized fact that the blood-sugar level may be influenced by the rate of absorption from the intestines. In animals in which the process of digestion is rapid and more or less intermittent, the blood-sugar level varies considerably throughout the day. After a meal of readily digested carbohydrates it increases for a time, but returns to a lower level as the rate of absorption decreases. To observe whether or not such a fluctuation occurred in dairy cows fed in the normal way, the blood sugar was determined at intervals during the day. The cows were fed at 6 a. m. and 4 p. m. Blood samples were taken at 7 and 10 a. m. and at 1, 3, and 5 p. m.

TABLE 3—*Variation in blood-sugar content in 22 dairy cattle at intervals throughout the day*

Hour of day	Time elapsed since feeding	Mean value for blood sugar
	Hours	Mgm per 100 c. c.
7 a. m. ....	1	51.4 $\pm$ 0.834
10 a. m. ....	4	49.0 $\pm$ 655
1 p. m. ....	7	50.7 $\pm$ 733
3 p. m. ....	9	52.9 $\pm$ 475
5 p. m. ....	1	50.5 $\pm$ 882

The results (Table 4) show a marked increase in blood-sugar concentration after dosage. This increase reached a maximum within approximately 2 hours, and 6 or 7 hours were required for the concentration to return to normal. In some of these trials it was possible to increase the blood-sugar content more than threefold.

That all this increase was not due to excitement resulting from the use of the stomach tube was shown by a repetition of the experiment in which water was used in place of the sugar solution. The average determination on four animals before the water was introduced was 65.3 milligrams of blood sugar. Thirty minutes after dosing the quantity had increased to 69.5 milligrams, one hour later it had decreased to 62.2 milligrams.

In every case the qualitative test (8) for sugar in the urine gave negative results for urine samples collected prior to the administration of glucose. Except in one trial, in which the blood sugar did not materially increase, sugar appeared in the urine within two to four hours after the solution was given. This glycosuria, which was to be expected under the conditions of the experiment, indicates that the "sugar threshold" value of the kidneys had been temporarily exceeded.

It is evident from these results that when soluble carbohydrate is given in such a way as to permit rapid absorption from the intestinal tract, a marked increase in the level of blood sugar results. This method of increasing the blood-sugar level has been used by the writers in studying the factors influencing lactose formation by the dairy cow.

#### EXCITEMENT

In order to determine the effect of excitement, the blood sugar of four cows was determined before and after a dog was brought into the barn. The nervousness of the cows resulted in an average increase in the blood sugar, ranging from 58.5 to 65.9 milligrams. When the dog was allowed to bark the blood sugar was further increased to 89.1 milligrams. All four cows showed a definite increase in this constituent.

Any undue excitement of an animal while the sample of blood is being taken for analysis may produce a marked increase in the blood sugar. This is particularly true when the animal is bled from the jugular vein. Unless one has had considerable practice in securing the blood in this manner, the animal may be rendered extremely nervous before the sample is finally obtained. In the work reported in this paper, the method used required only 0.1 cubic centimeter of blood, which could be taken quite conveniently from the ear, with a minimum of discomfort to the animal. The uniformity of the results recorded in Tables 1 to 4 may probably be attributed to this and the uniform conditions under which the samples were taken.

#### OESTRUS

A limited number of observations were made on cows during oestrus. In each case studied there was a distinct rise in the blood sugar, amounting in two instances to as much as 40 per cent. Hewitt (7) has reported blood-sugar values as high as 362 milligrams for heifers during oestrus. Observations made on five heifers in this herd gave no value in excess of 90 milligrams.

The solutions were administered at 9 a m, immediately after an initial blood sample had been taken. Samples were then secured at 30 minutes, 1, 2, 4, and 7 hours after dosage. In the first two trials the animals had been deprived of feed for 12 hours. In the remainder

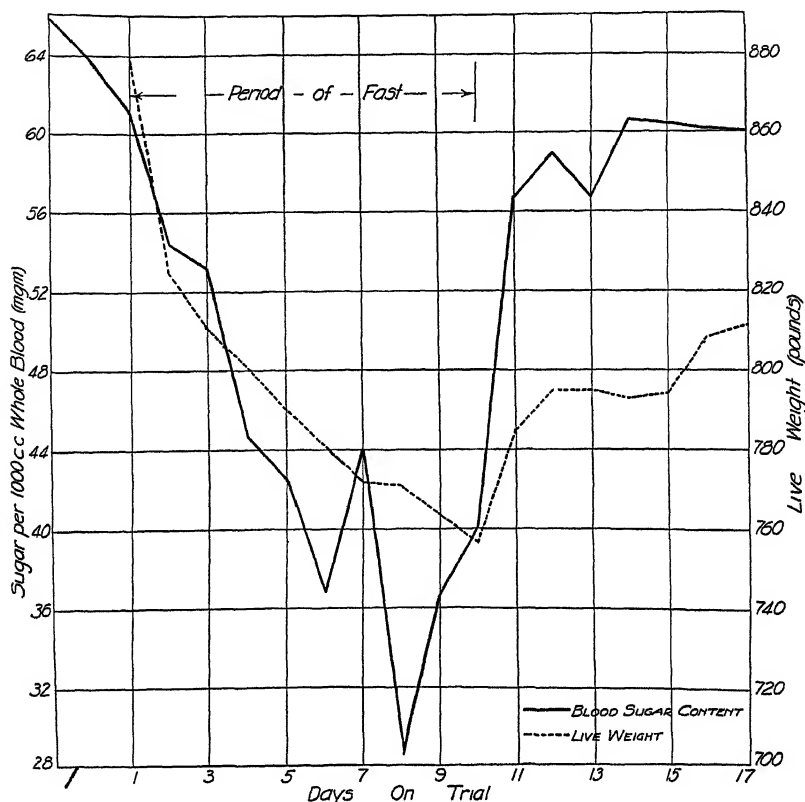


FIGURE 1.—Influence of fasting on the blood-sugar level and live weight of dairy cattle. The sudden rise in blood sugar on the seventh day was undoubtedly caused by the fact that the heifers broke through the fence and obtained some roughage the evening before.

no attempt was made to withhold any part of the regular ration prior to giving the sugar.

TABLE 4.—Effect of the administration of glucose on the blood-sugar level of dairy cows

Breed and daily milk record of animal	Glucose administered	Milligrams sugar per 100 cubic centimeters whole blood at time indicated					
		Before dosage	30 minutes after dosage	1 hour after dosage	2 hours after dosage	4 hours after dosage	7 hours after dosage
	Pounds						
Holstein, dry.....	8	43 1	108 7	133 3	156 2	114 9	54 0
Holstein, dry.....	6	47 8	78 7	69 0	105 3	91 0	59 5
Guernsey, 9 pounds.....	7	67 6	84 7	94 3	133 3	64 5	52 6
Guernsey, 10 pounds.....	7	50 0	84 0	113 6	166 7	52 4	50 0
Holstein, 40 pounds.....	8	59 2	137 0	166 7	88 5	83 3	63 3
Guernsey, 25 pounds.....	6	54 0	117 6	105 3	100 0	76 9	58 8
Guernsey, 24 pounds.....	8	50 0	77 5	100 0	90 0	71 9	57 1

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## TEMPERATURE

As already stated, the results recorded in these studies were secured during the winter months, when the cows stayed in the barn a large part of the time and were maintained under uniform conditions. Some determinations made since in another study during extremely warm July and August weather gave results considerably higher than any observed heretofore. Further investigation will be required to determine definitely whether the increase in the blood-sugar level was due to the high temperature prevailing at the time the tests were made or to other factors.

## SUMMARY

In the course of this investigation, blood samples from 140 dairy cattle were analyzed for sugar content. The following results were obtained:

Calves shortly after birth had a blood-sugar content of about 100 milligrams per 100 cubic centimeters. As the age of the animal increased the blood sugar decreased until the animal was approximately 2 years of age, after which little further change was observed. A mean blood-sugar content of  $53.03 \pm 0.297$  milligrams per 100 cubic centimeters of blood was obtained on 222 samples from 74 animals between 2 and 8 years of age, with a range of 35 to 74 milligrams.

No significant difference was observed in the blood-sugar level of the four breeds studied.

Cows giving a liberal flow of milk were found to have slightly less blood sugar than dry cows or those yielding a small quantity of milk.

There was no increase in blood sugar after feeding. A slight increase was observed at 3 p. m., which probably may be accounted for by the increased activities about the barn prior to the afternoon feeding.

Fasting caused a decrease in the blood-sugar content of dairy heifers amounting approximately to 50 per cent.

The administration of glucose in solution produced increases in the blood-sugar content amounting to as much as 200 per cent.

Excitement was found to produce a marked increase in blood sugar.

The blood-sugar values of cows and heifers were higher during oestrus than at other times.

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# THE GERMINATION OF COTTONSEED AT LOW TEMPERATURES <sup>1</sup>

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## INTRODUCTION

One of the most important factors in the production of a large crop of cotton is a full stand of plants early in the season. Studies concerning the effect of date of planting on yield carried on at the South Carolina Experiment Station for the past six or seven years have shown that the cotton planted earliest produces the highest yield if a permanent good stand is secured. But the period of greatest mortality of cotton plants due to adverse environmental conditions is from the time the seed is planted until the seedling stage is past. It is during this time that cold wet weather so often makes early planted cotton a failure. It follows, therefore, that if a variety of cotton, excellent in other respects, could be found with more resistance to cold in the seedling stage, farmers would be able to plant earlier or would be assured of better stands from plantings at the usual time. In either case they would secure greater yields.

The isolation or development of such a variety by means of field selections is not easy. One season may be suitable for such selections and the next one totally unsuitable. It is therefore very desirable to devise a better method for making the selections, if it can be done. A possible method of doing this was suggested by results secured with corn by B. D. Leith,<sup>3</sup> of the Wisconsin Agricultural Experiment Station. By a method which consisted in part of testing the seed in a refrigerator and selecting for planting only those ears from which germinations occurred within a given time, he was able to produce a harder strain of corn than the earliest then grown in Wisconsin. As part of an attempt to adapt this method to the selection of cold-resistant strains of cotton, a study of the germination behavior of the seed of a number of varieties was made. The chief results are reported in this paper.

## METHODS

Briefly stated, the method used in these experiments was to germinate samples of the best seed of different lots at approximately the minimum temperature at which germination would occur, and then to compare the behavior of the different strains and individuals. The percentage germination was not of itself considered especially

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perature readings were made immediately after the trays were removed from the incubator for examination. During the course of the examination the temperature inevitably rose somewhat, since the trays were necessarily exposed to the warmer air of the room during that time. There were also a few occasions when the refrigerating system failed to operate properly. During the first year in particular this happened more often in the later, warmer part of the season than earlier. For this reason, and because the air to which the seeds were exposed during observations was warmer in the later part of the season, the effective temperature under which germination occurred was higher each season when the last lots were germinated than when the first were germinated. In order to get a measure of the effect of these environmental variations, a strain of Cleveland (lot No 1, Tables 1 and 2) was selected as a standard and a sample was included with every group of samples germinated. It proved to be a very high grade lot of seed from the standpoint of germination at the higher temperatures. Some of the samples gave perfect germination and none failed to give a very high one.

Enough water was sprinkled on the covering paper after the daily examination had been made to make it appear as moist as at the beginning of the experiment. Tests were conducted to determine whether the inaccuracies of this method of replacing evaporated water were such as to introduce significant variations in the percentage of germination. The results showed that too much or too little water would decrease germination, but the variations in water content involved in these tests were large enough to be easily detectable by appearance. As a matter of fact, the paper very quickly took on a slightly dry appearance if the level of free water fell below the surface of the sand, and became soaked with water if the level rose above the surface. There seems to be no reason to suspect that better germinations could have been secured with a different amount of water in the sand or that the variations in moisture were sufficient to influence the results appreciably.

After samples had been kept at the low temperature until no more germinations occurred or until other circumstances made it necessary to discontinue the test, they were placed at the high temperature, 25° to 30° C, for a time to determine how many, if any, of the remaining seeds were still alive.

The earliest germinations were carried out in a small, improvised incubator composed of a wooden box set in an ice-cooled refrigerator and equipped with electric heating units and a very simple thermostat. The work in the early months of 1926 was done in the laboratories of plant physiology at the University of Illinois. The constant-temperature chambers built and operated by C. F. Hottes<sup>5</sup> were used in these tests. The last two seasons' germinations were made at Clemson College in a constant-temperature chamber built for the purpose. It consisted of an insulated box placed in an electric refrigerator and equipped with heating coils, thermostat, and air stirrer. With this apparatus it was possible to get satisfactory

<sup>5</sup> HOTTES, C. F. A CONSTANT HUMIDITY CASE (Abstract) *Phytopathology* 11:51 1921.

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significant. The information desired was what the viable seed would do at the low temperature. For this reason all apparently defective or damaged seeds (lightweight, pale, gin cut, etc.) were discarded, and parallel tests of all samples at 25° to 30° C were run. The higher temperature was assumed to be near the optimum, and, while it was not definitely so proved, the promptness and high percentage of germination repeatedly secured indicated that the assumption was not far wrong. It is clear, for instance, that a variety germinating 50 per cent at the low temperature would be distinctly superior from the standpoint of resistance to cold to one germinating 75 per cent, if the respective germination rates at the optimum should be 50 and 95 per cent, respectively. In the first case 100 per cent of the viable seed would germinate at the low temperature while in the latter only 79 per cent would do so. The germination percentages reported at the low temperatures are therefore based on the number of seeds which the test at the high temperature showed to be viable.

The germinations reported are for time intervals commencing with the beginning of the test and ending with the close of the week mentioned. Since in a study of this kind the number of seeds which germinate, say, by the end of the second week, is more important than the number which germinate during the second week of the period, the germination percentages by individual weeks are not given. The values are not always given in detail after the fourth week, although the experiments were often conducted longer.

Preliminary experiments to determine the most suitable method of germinating the seed included germination on plaster of Paris blocks in water and in wet sand, both with and without a cloth or paper covering; germination in a shallow layer of water, and germination between moist absorbent papers. The last method was the most satisfactory, and the best plan found for keeping the papers properly moist was to lay the lower one on a uniform layer of sand about one-half inch deep saturated with water in a covered, ventilated tray or pan. The suitability of delinting the seed with sulphuric acid as compared with the prewetting method of Toole and Drummond<sup>4</sup> was also investigated. The delinted seeds, as shown in the first part of Table 1, germinated better, partly no doubt because delinting made it possible to detect and remove most of the defective seeds. Delinting was therefore adopted as the method of preparing the seeds for the tests.

Examinations were made daily so far as possible. Temperatures were taken by means of thermometers lying on the upper layer of paper, germinated seeds were removed and counted, and water was added to replace that lost by evaporation. In the earlier part of the work it was found difficult after opening a tray to get the thermometer reading before the warmer air of the room had caused it to change. Later this difficulty was overcome by tying some filter paper around the bulb. With this treatment there was abundant time for noting the reading before any change occurred.

It should be noted that the actual effective temperature was really somewhat higher than that observed, even when the readings were most accurately taken. This was owing to the fact that tem-

<sup>4</sup> TOOLE, E. H., and DRUMMOND, P. H. THE GERMINATION OF COTTONSEED. *Jour. Agr. Research* 28, 285-291, illus. 1924.

TABLE 1.—Comparative germination of cottonseed at 12°, 15°, and 25°–30° C

Lot No.	Variety	Source	Treatment	Date of start	Germination at 12°										Germination at 15°										Germination at 25°-30°																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
					Seeds used	Seeds germinated after—				Total germinated	Germinated later at 25°	Seeds killed	Seeds used	Seeds germinated after—	Total germinated	Germinated later at 25°	Seeds killed	Seeds used																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
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2	Delfos 631	Delta Experiment Station, Stoneville, Miss.	Delinted	Feb 19	No	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	1

\* Fungal infections so severe that 12° and 15° lots were discarded in the middle of the third week

control and practical uniformity of the temperature. Thermometer readings indicate that there may have been at times a maximum of 0.3° C difference in temperature between the bottom tray and the top one. In order to equalize the effect of any such difference the position of the trays in the incubator was changed regularly.

## RESULTS

### GERMINATION BEHAVIOR AT LOW TEMPERATURES

Preliminary experiments made with extemporized equipment in 1925 with a few varieties of upland cotton indicated that the minimum temperature for germination is approximately 12° C. The results of the later experiments are shown in detail in Tables 1 and 2. While not permitting the precise determination of the minimum temperature for germination, they fully confirm the original results in a general way. They indicate that cottonseed might possibly germinate at slightly lower temperatures if given enough time, but that it is far from likely that it would germinate at an appreciably lower temperature than 11°, especially since, as already explained, the actual effective temperature in the tests was slightly above that recorded. Certainly the germinations would not proceed at a lower temperature at a rate that would make such tests practical for selection work.

	28	29	30	31	32	33	1	34	35	36	37	38	39	40	41	42	43	44	45	46
Belton, T S No 5984-91	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Texas Agricultural Experiment Station (Substation No. 5), Temple, Tex	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Lankford Seed Farm, Waco, Tex	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Bureau of Plant Industry grown at San Marcos, Tex	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Bureau of Plant Industry, grown at Greenville, Tex	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
J W Davidson, McKinney, Tex	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Piedmont Pedigreed Seed Farm	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
South Manchuria	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Gossypium Nanking, Manchurian Black Seed Kuro-tane, C B	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
G Nanking, Manchurian White Seed, Shiro-tane, C B No 478	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
C B 292	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Tree cotton, C B No 280-A	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Peruvian tree cotton, C B 240-C	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Caravaca, C B No 243	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Caravaca hybrid C B 127-B	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
San Lucas cotton, C B No 229	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Varca (white), C B No 204	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Varca (brown), C B No 204	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Florida cotton, C B No 160	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
C B No 185-1/2 B	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Italian cotton	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
Violet, C B No 244	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do	do
100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr	Apr
1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0
6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2	6 2
71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1	71 1
2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74	2 74
11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3	11 3
14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4	14 4
200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200
21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1	21 1
89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7	89 7
91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2	91 2
1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5
7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2	7 2
200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200
97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0	97 0

\* The fourth week in these instances was only 6 days  
 \* The second week in these cases is 6 days, third week 8 days, and fourth week 6 days  
 \* The second week in these cases is 6 days, third week 8 days, and fourth week 6 days  
 \* The fourth week is only 1 day, the character reached 16° on the thirteenth day and 20° on the sixteenth day  
 \* Transferred to 25° chamber at end of third week  
 \* Left 4 days only at 25°

From the Office of Acclimatization and Adaptation of Crop Plants, Bureau of Plant Industry Grown at their acclimatization gardens at Torrey Pines in south California  
 Numbers refer to their breeding numbers.  
 † No seeds put to germinate, owing to small size of sample available

TABLE 1—Comparative germination of cottonseed at 12°, 15°, and 25°–30° C.—Continued

Lot No	Variety	Source	Treatment	Date of start	Germination at 12°										Germination at 15°										Germination at 25°-30°						
					Seeds used	Seeds germinated after—				Total germinated	Germinated later at 25°	Seeds killed	Seeds used	Seeds germinated after—				Total germinated	Germinated later at 25°	Seeds killed											
						1 week	2 weeks	3 weeks	4 weeks					1 week	2 weeks	3 weeks	4 weeks														
15	Delta and Pine Land No 4	Delta & Pine Land Co of Mississippi, Scott, Miss	Delinted	1928 Mar 16	No 200	Pd 9.7	Pd 41.0	Pd 52.3	Pd 60.0	Pd 10.5	Pd 20.5	No 200	Pd 7.2	Pd 53.9	Pd 70.0	Pd 87.7	Pd 4.1	Pd 8.2	No 200	Pd 7.2	Pd 53.9	Pd 70.0	Pd 87.7	No 200	Pd 7.2	Pd 53.9	Pd 70.0	Pd 87.7	Total germinated	Seeds used	Total germinated
16	Snow Flake	John McGlendon, Clarks-ville, Tex	do	do	200	7.6	43.7	52.8	59.9	11.7	28.4	195	2.1	75.5	84.9	90.1	4.2	5.8	200	2.1	75.5	84.9	90.1	5.8	200	2.1	75.5	84.9	90.1	98.5	
17	Covington Toole	Florida Agricultural Experimental Station	do	do	200	7.5	53.5	62.0	69.0	5.0	25.1	200	6.4	87.7	92.0	(*)	92.0	2.1	5.9	200	6.4	87.7	92.0	5.9	200	6.4	87.7	92.0	93.5		
18	New Boykin (stock No 832)	Ferguson Seed Farms, Sherman, Tex	do	do	200	5.8	22.6	37.4	48.4	36.9	14.7	200	3.7	67.9	86.8	90.6	91.6	3.7	4.7	200	3.7	67.9	86.8	90.6	4.7	200	3.7	67.9	86.8	95.0	
19	Mebane	A. D. McBane Sales Agency, Lockhart, Tex	do	do	200	4.3	32.8	35.5	35.5	5.6	64.0	200	48.4	64.5	64.5	64.5	5	34.9	200	48.4	64.5	64.5	5	34.9	200	48.4	64.5	64.5	93.0		
20	Startex No 333 (a selection from Lone Star)	Texas Agricultural Experiment Station, College Station, Tex	do	do	200	3.9	35.6	46.1	48.3	16.7	50.0	200	72.2	81.1	82.2	82.2	0	17.8	200	72.2	81.1	82.2	0	17.8	200	72.2	81.1	82.2	90.0		
21	Name unknown	(Donor not known), Rocky Mount, N. C.	do	Mar 16	200	1.6	28.6	45.3	55.2	20.3	24.5	200	2.7	73.4	88.0	93.2	1.0	5.7	200	2.7	73.4	88.0	93.2	1.0	5.7	200	2.7	73.4	88.0	96.0	
22	Carolina Foster	Humphrey-Coker Seed Co., Harts-ville, S. C.	do	do	200	1.6	11.8	19.3	20.9	8.6	70.6	200	1.1	24.6	46.0	47.6	2.1	6.0	200	1.1	24.6	46.0	47.6	2.1	6.0	200	1.1	24.6	46.0	93.5	
23	Super Seven	Pedigreed Seed Co., Harts-ville, S. C.	do	Mar 15	200	33.2	43.4	56.6	18.9	29.6	200	40.3	77.0	83.7	83.7	6.0	9.7	200	40.3	77.0	83.7	83.7	6.0	9.7	200	40.3	77.0	83.7	98.0		
1	Cleveland	Piedmont Pedigreed Seed Farm, Commerce, Ga	do	do	201	6.5	16.4	25.4	45.8	28.8	200	4.5	34.0	56.5	56.5	29.0	14.5	200	4.5	34.0	56.5	56.5	29.0	14.5	200	4.5	34.0	56.5	100.0		
24	Pima	Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C., grown in Arizona	do	Apr 1	200	38.8	40.6	90.3	93.4	0	6.6	200	96.4	98.5	98.5	98.5	0	1.5	200	96.4	98.5	98.5	0	1.5	200	96.4	98.5	98.5	98.0		
25	Sea island	Bureau of Plant Industry, grown near Charleston, S. C.	do	do	200	36.1	39.1	39.1	39.1	0	60.9	200	33.8	45.6	54.1	54.1	0	45.9	200	33.8	45.6	54.1	54.1	0	45.9	200	33.8	45.6	54.1	66.5	
26	Rowden	Bureau of Plant Industry, grown at Wills' Point, Tex	do	do	200	5.1	4.0	50.0	62.5	3.3	34.2	200	56.5	84.2	87.5	88.0	0	12.0	200	56.5	84.2	87.5	88.0	0	12.0	200	56.5	84.2	87.5	92.0	
27	Kekchi	Bureau of Plant Industry, grown at Clarks-ville, Tex	do	do	200	1.0	6.3	42.9	79.6	15.2	5.2	200	26.7	45.3	82.2	91.1	2.6	6.3	200	26.7	45.3	82.2	91.1	2.6	6.3	200	26.7	45.3	82.2	95.5	

24	Prima, 1925 crop	Bureau of Plant Industry grown in Arizona	Apr	29	11	7	200	1	61	42	7	51	6	52	1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---</
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<sup>a</sup> Germination test made in February used as a control here



sections where both Pima and upland varieties are grown Pima is planted earlier<sup>6</sup>

The second class of cotton varieties investigated includes all those not already mentioned, with the possible exception of some of the foreign varieties of which only a few seeds were available. In this group there is apparently a considerable variation between varieties, although it is practically impossible to give any of them more than an approximate place in an arrangement based on the performance in question. An attempt has been made in the tables to make such an approximate arrangement for each separate group studied. Since it was found impossible to maintain conditions from one germination test to another such that the germination of the check variety would remain constant, and since there were likewise a number of as yet unexplained variations in the relative germinations of identical lots of seed, no attempt has been made to classify the group as a whole. About the only statement that it seems safe to make is that the strain

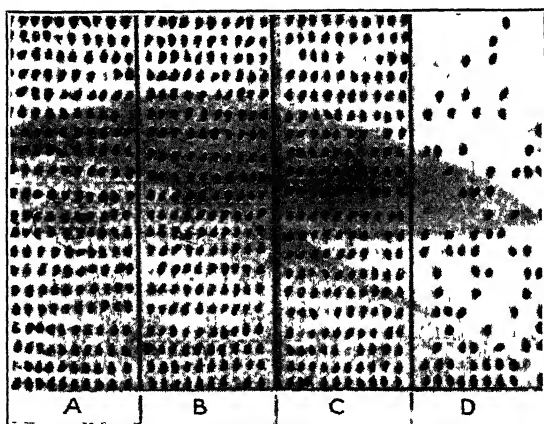


FIGURE 1.—Germination of seeds of Rowden (A), Kekchi (B), sea island (C), and Pima (D) cotton after about 10 days at 12° C. The samples originally contained 200 seeds each. Vacant places represent seeds which germinated and were removed. Test started April 1, 1926.

of Cleveland used as a check stands well toward the foot. Cleveland is considered one of the earlier varieties, but its earliness, at least in so far as this strain is concerned, is apparently not due to ability to germinate and grow well in cool weather.

In this connection it is worth noting that failure to germinate at the low temperature does not necessarily mean that the seeds were killed (Columns 12, 13, 20, and 21 of Table 1 and columns 17 and 18 of Table 2). This particular lot of Cleveland seed, for instance, always had a large percentage of viable seed remaining after a month or more at each test until the last, at which time it was over 2 years old. Such a characteristic would often permit early planting, even if the weather were not at once suitable for germination. Whether this character is hereditary or merely a chance characteristic of the samples in which it was observed can not now be stated.

<sup>6</sup> Communication by C. B. Doyle, Bureau of Plant Industry, U. S. Department of Agriculture.

A second point worth noting here is that near the minimum for germination a small difference in temperature makes a much greater difference in the behavior of the viable seeds than it does at a higher temperature. Thus the unweighted mean of the total germinations at 12° C as given in Table 1, omitting the foreign cottons represented by only a few seeds each, is 30.7 per cent, while that at 15° is 71.7 per cent. Similarly, the means for the seeds killed by the treatment are 51.4 and 23.4. In other words, reducing the temperature from that used as a control (25°–30°) to 15° reduced germination only 28.3 per cent (100–71.7 per cent), while reducing it 3° further reduced it 41.0 per cent (71.7–30.7 per cent). Likewise reducing the temperature to 15° killed 23.4 per cent of the viable seeds while the 3 degrees additional reduction killed 28.0 per cent more. The relative speed of germination, while not so easily summarized in a few figures, is very similar, as can be seen from Table 2. Another consideration showing the great variation in response resulting from a small change in temperature in this range is the fact that it was found practically impossible to repeat experiments with conditions enough alike to get identical results within the range of natural variation expected between samples. Any temporary failure of the regulatory apparatus to function, allowing the temperatures to rise for a short time, or apparently even merely the variation in room temperature where the daily examinations were made was enough to produce a significantly different behavior in the germination. Thus, owing to the slightly higher effective temperature in the later part of the season during the first two years, as explained under Methods, the germination became more rapid as the season advanced. While these results do not prove the point, they suggest rather strongly that the temperature coefficient for cotton germination in this range is considerably greater than at higher temperatures.

#### VARIETAL VARIATIONS

Reference to Tables 1 and 2 show that the varieties studied fall into two distinct classes with regard to the rate and percentage germination of the seed at temperatures near 12° C. In one class the rate is distinctly greater than in the other. The more rapidly germinating class includes the American-Egyptian variety, Pima, and two varieties, Manchurian Black Seed and Manchurian White Seed, of the Asiatic cotton, *Gossypium nanking*. There can be no question about the inclusion of Pima and Manchurian Black Seed in this group, as they were tested at different times with results that were consistent throughout. There is more question with regard to Manchurian White Seed, since in this case the placing is made on the basis of the preformance of only 35 seeds. However, in the one test made with the variety its behavior was so similar to that of Manchurian Black Seed that there seems to be no reason why it should not be placed in the same group. Figure 1 shows the germination of Pima in 1926 as compared with three other varieties after about 10 days in the germinator and Figure 2 shows the germination of Manchurian Black Seed as compared with College No. 1 in January, 1928. In examining these illustrations it should be recalled that the seeds were removed as soon as they germinated, so that a blank space represents a germinated seed. It may be mentioned in this connection that the finding with regard to Pima is in line with the general experience of farmers in the Southwest. In

is clear that the seed was affected adversely by weather conditions while the crop was maturing. The effect was of such a nature as not to be detectable under conditions favorable for germination but easily detectable at low temperatures.

The considerations mentioned in the last two paragraphs may explain at least in part some of the contradictory results obtained with most of the upland varieties. However, there seems to be no reason for thinking them important enough to bring into question the finding concerning Pima, *Gossypium nanking*, or the strain of Cleveland mentioned. The performance of these strains was consistent throughout and extended through two generations in each case.

#### INDIVIDUAL VARIATIONS

Further reference to Tables 1 and 2 discloses the fact that there are among the seeds of most, and probably all, varieties a few that will germinate a week or more before the bulk of the germination occurs. Whether this earlier germination has a genetic basis or is only the expression of chance environmental conditions can only be learned by breeding trials, which in the present case have not gone far enough to determine this point. In the case of Pima and other pure-line varieties, the latter is more apt to be the case than with the more heterogeneous varieties. Although a pure-line variety may not have been selected for the character in question, still the repeated selfing employed in stabilizing it must have led to gametic purity in this regard as in others. However, the data indicate that variations occur often enough in the direction of increased ability to germinate at low temperatures to make comparatively easy the isolation by selection of strains notably strong in this regard, provided of course that such variations are in fact heritable. Whether the isolation of such strains can be considered worthwhile will depend, of course, on whether the ability in question is correlated with hardiness to cold in the seedling stage.

#### EFFECT OF AGE OF SEED ON GERMINATION IN THE COLD

As suggested above, age may affect the germination of cottonseed in the cold. It is very difficult to arrange experiments to test this hypothesis, owing to the great difficulty of maintaining exactly the same temperature conditions for the germination of successive lots of seed and to the relatively large effect which small temperature increments exert near the minimum. For this reason the comparative germination rates of successive samples of identical lots of seed can throw little light on the problem.

However, during the winter of 1926-27 old seeds of Pima and College No. 1 were twice tested in comparison with seeds of the same varieties of the succeeding season's crop (Table 2). With Pima the advantage in the first test was clearly with the younger seed, both as to rate of germination and number which germinated, in the second test the rates were practically the same. With College No. 1 a larger early germination was exhibited in both cases by the older seed, and in the first trial a greater total germination as well. In the second trial the total germination was about the same for the two lots.

In 1927-28 some 1925 seeds of Piedmont Cleveland were tested in comparison with selfed seeds grown locally in 1927 from the same lot. The older seed germinated more promptly. Figure 3 shows the two lots of seed on the twenty-fourth day after they were put to germinate.

A possible explanation of some of the contradictory results mentioned above lies in the fact, to be discussed below, that some of the data suggest that the germination performance of cottonseed varies after a year or so. If this is true, the relative varietal performance might vary considerably from time to time, depending on the relative age at which seed of the different varieties undergoes the change. In this case, of course, it would be necessary to make varietal comparisons before the seed of any had undergone the change mentioned. Presumably almost any time within the first year after harvest would be safe.

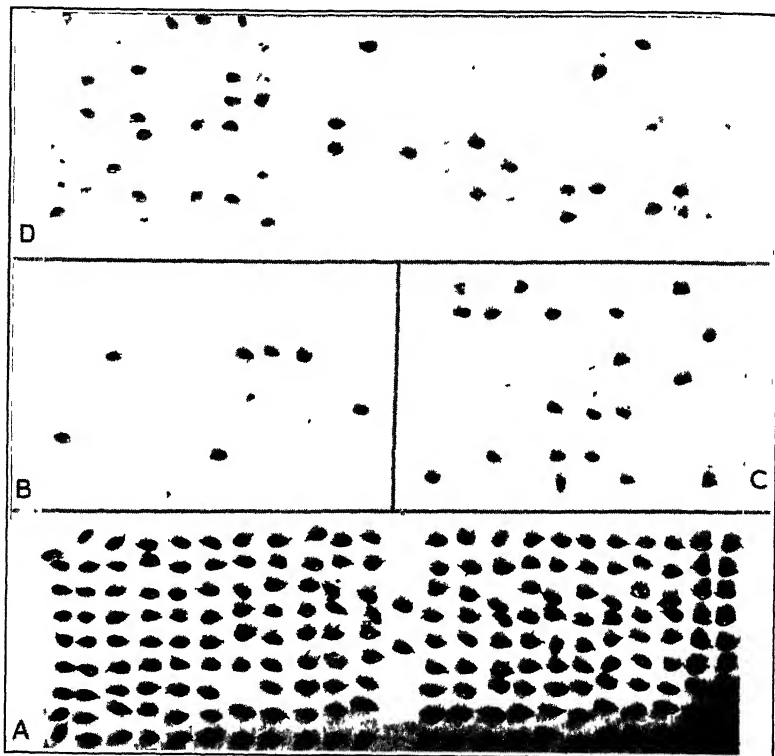


FIGURE 2—Germination of seeds of College No. 1, lot 62 (A), *Gossypium nanking*, C B 477, lot 56 (C-D), and of a selection of the latter after 24 days at 12° C. Samples originally contained 200, 300, and 100 seeds, respectively. Seeds in (B) not considered in Table 2. Seeds grown at Clemson College in 1927, test started December 16 and 17, 1927.

It is probably true also that for a critical comparison of varieties where the difference is not very marked the seed should be grown in the same locality under as nearly identical conditions as possible and should be kept under identical storage conditions after picking. It is interesting to note in this connection that in all cases where Kentucky-grown seed was tested in comparison with seed of the same variety grown elsewhere (Tables 1 and 2) the seed grown elsewhere germinated more quickly at low temperature. But, owing to drought and extremely high temperature, the cotton from which the Kentucky-grown seed was taken opened prematurely. Thus, the indication

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## SOME FACTORS ASSOCIATED WITH THE BREEDING OF ANOPHELES MOSQUITOES<sup>1</sup>

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### INTRODUCTION

The fact that in a given locality *Anopheles* is found breeding in certain water areas and not in others, or sparsely in some areas and abundantly in others, has given rise to considerable conjecture and no little investigation as to the causes of this condition. The present paper summarizes the results of two seasons' observations in the vicinity of Mound, La. This locality is in the northeastern part of Louisiana in what is known as the Louisiana Delta region. Here the land is more or less flat, which accounts for the poor natural drainage and gives rise to numerous shallow lakes in the lower areas. All of these areas are, to a greater or less extent, covered by tree growths. The streams of the region are known as bayous. They are sluggish, having little or no current except after heavy rains, and are usually more or less overgrown with trees and brush, except in such stretches as have been cleared of this material. Land-locked branches of bayous form sloughs which are similar to the bayous except that they are stagnant throughout the year and usually contain larger quantities of aquatic and semiaquatic vegetation. These areas, all of which produce *Anopheles* to a greater or less extent, have been described in a previous paper (3).<sup>2</sup>

### METHODS OF OBSERVATION

Observations on the occurrence and abundance of *Anopheles* at selected points, or "stations," in these water areas were made monthly from May to September, inclusive, during 1928 and 1929. The larval abundance rate was determined on the basis of the number of larvae taken in a collection of 10 dips of surface water, the dips being made with an ordinary white-enameled water dipper about 5 inches in diameter. At the time of each collection observations were made on the environmental conditions in the area, and the hydrogen-ion concentration of the surface water was determined. A sample was taken of the top one-half inch of the water and this was carried immediately to the laboratory, where, after thorough shaking, 1 c c was placed in a Sedgwick-Rafter counting cell and a count made of the number of organisms in 40 c mm of water, as a basis for computing the rate of occurrence of the organisms per cubic centimeter in the water. After this count the organisms in a large

<sup>1</sup> Received for publication Aug. 10, 1931, issued April, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 399.

The older sample, at the higher temperature, germinated more poorly than previously. What may perhaps be interpreted as confirmatory evidence is the fact, already mentioned, that the placement of varieties differed rather widely from test to test. This result would be expected if the seed of one variety passed through the change more rapidly than that of another.

On the whole, while the data can not be claimed to prove that an improvement in the ability of cottonseed to germinate in the cold

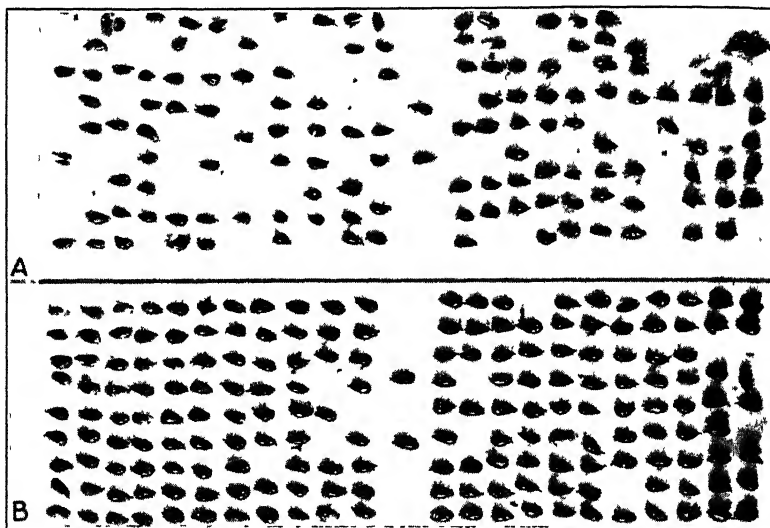


FIGURE 3.—Germination of 3-year old, lot 1 (A), and last season's seed, lot 59 (B) of the same strain of Cleveland cotton after 24 days at 12° C. Younger seed grown at Clemson College. Test started December 17, 1927.

occurs as the seed becomes older, they do suggest that a slight improvement may occur which is distinct from anything in the nature of afterripening.

#### SUMMARY

The minimum temperature for the germination of cottonseed is approximately 12° C. The increase in activity up to 15° is rapid. Seeds that do not germinate at a given low temperature will often remain viable as long as two months or more under the unfavorable conditions.

Of all the varieties studied Pima and two varieties of *Gossypium nanking* exhibit the most rapid and most complete germination at low temperature. A strain of Cleveland used repeatedly ranks well toward the foot. Most of the upland varieties studied are intermediate, but owing to conflicting evidence it is impossible to rank them among themselves.

There are individual variations in the ability of cottonseed to germinate in the cold, which if hereditary and correlated with seedling hardiness, can be used in selecting for this latter character.

There is some indication that the rate and percentage of germination in the cold increase with the age of the seed, at least for a year or two.

of which are devoid of aquatic vegetation the production of *Anopheles* ceases

In the data given in Table 1 it is seen that the surface-water index for 1928 fell only gradually from May to August and was accom-

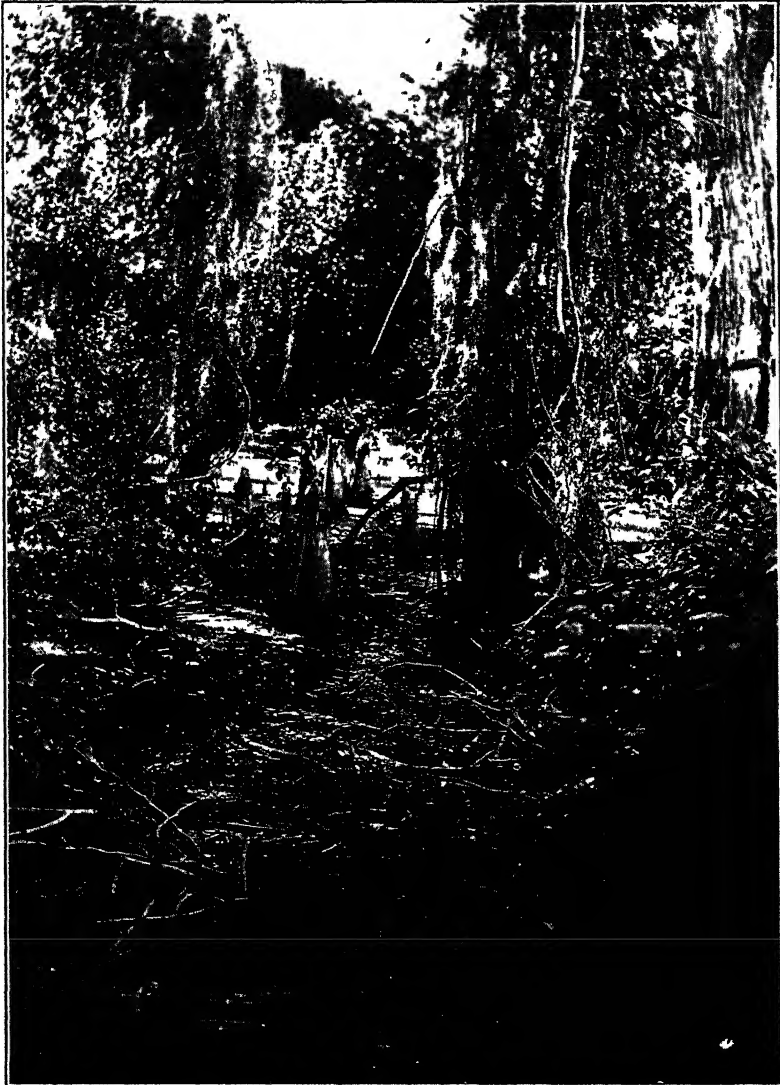


FIGURE 1—An *Anopheles* breeding place at the edge of a swamp lake. Note how the débris is becoming stranded as the water level grows lower

panied by a rising larval rate. A rapid diminution of the water surface then occurred from August to September, attended by a falling larval rate. In 1929 the surface water decreased rather rapidly from May to August, as did the larval abundance rate. From August to

sample of the water were concentrated by either filtering or centrifuging and the sediment was examined for the presence of other forms. Samples of algal patches and algae-coated sticks were also collected and examined.

In these examinations determinations, as far as genera when possible, were made of the forms observed, but those not readily fitting into the keys available were grouped only into classes. The *Anopheles* larvae collected were taken, in water from the breeding area, to the laboratory, determined as to species, and dissections made of some, usually five, of the larger specimens in order to ascertain the nature of the food ingested. In only two instances were species other than *quadrifasciatus* found, and these were taken from areas where *quadrifasciatus* also occurred. In these two cases the gut contents were similar for all species.

#### INFLUENCE OF TEMPERATURE, PRECIPITATION, AND QUANTITY OF SURFACE WATER UPON ANOPHELES LARVAL ABUNDANCE RATES AND PLANKTON CONTENT OF THE WATER

In summarizing the observations it has been assumed that temperature and other conditions influencing the activity of *Anopheles* during the five months from May to September, inclusive, are sufficiently constant to make all observations comparable. Table 1 gives a summary of the larval collection records and plankton counts for the two seasons, arranged by months, together with pertinent meteorological data and a surface-water index. This index is the average of the width in feet of the water area at the various observation stations, computed or measured when the larval collections were made. The records on plankton findings for each year are divided into two groups—those for the breeding stations where *Anopheles* usually were found, and those at stations located in impounded bayou areas where, on account of special conditions, *Anopheles* larvae were consistently absent.

The figures for 1928 show the monthly larval rate as having a seasonal rise and fall with the rise and fall of the mean air temperature, the highest rate being in August, when the highest mean temperature occurred. For the following year, however, there are two peaks in the larval rate, one in May and the other in September, in which months the lowest mean temperatures occurred. The writer has found from observations in this locality extending over a number of years that the variation in summer temperatures does not noticeably affect larval population as measured by dipping. In a previous paper (3) the larval rates obtained during three other seasons are given and further illustrate this fact.

The summer rainfall in this region is not usually sufficient to keep the surface-water areas from showing a steady decrease as the season advances. This gradual decrease in water surface tends to keep the margins of the water areas more or less free from vegetation, which encroaches on the shore line in times of stationary or rising water, and to remove from the surface of the water large quantities of floatage which are blown to the margins by the wind. This is not refloated, except in case of exceptionally heavy rains, until the high waters of the ensuing winter. (Fig. 1) The lowering of the water level also gradually leaves the tree and brush covered marginal areas, particularly those of the lakes, dry; and in the lakes the central parts

Considering next the effect of temperature, surface water, and rainfall on plankton organisms (Table 1), it appears that there is a wide range of variation when the data for a like group of stations for the two years or for unlike groups during the same year are considered. The impounded-bayou group of stations in both years shows as a rule greater abundance of plankton than is shown by the breeding group. (Tables 1 and 2) This condition is associated with clear water surfaces and absence of shade (Fig 2) An increasingly greater



FIGURE 2—View along a cleared and impounded bayou, showing absence of debris and vegetation. Such places as this are mosquito-free

number of plankton organisms appears to be present in the water as the season advances and as the volume of water diminishes, but this increase is quite irregular and is caused no doubt by the common phenomenon of periodicity in certain species of organisms. On four different occasions one collection was found to contain so large a number of one kind of organism that it colored the entire body of water at the station where it was taken. The high counts obtained in these instances unduly affect the entire average and for comparative purposes are better omitted. The averages obtained when these high counts are included are shown in braces in Tables 1 and 2.

September, however, there was little fall in the surface water, making for more or less stable conditions in the breeding areas, accompanied by a rise in the larval index. These conclusions are of course applicable only to conditions as they occurred in this locality during the two years under consideration, for it is known that under certain conditions the opposite may be true, that is, a lowering water level may increase larval abundance by making large central open areas in lakes suitable for the breeding of *Anopheles*. One example may be cited of a large lake in which the early spring breeding was entirely marginal. The bed of this lake was almost completely covered by a thick growth of *Potamogeton* and *Chara*, which early in the season did not reach the surface. As the water became shallow, the long stems of these plants came to lie just at the water surface, providing excellent protection for *Anopheles* larvae and giving rise to the most intensive breeding over hundreds of acres of water surface.

TABLE 1—*Larval rates of Anopheles and plankton counts compared to temperature, precipitation, and surface water under at Mound, La*

(One collection of 10 dips made at each station each month)

#### BREEDING STATIONS

Year and month	Number of stations	10-dip larval rate	Precipitation	Surface water index	Temperatures			Average number of organisms per cubic centimeter									
					Maximum	Minimum	Mean	Total plankton	Flagellates	Ciliates	Amoeboids	Diatoms	Chlorophyceae	Cyanophyceae	Miscellaneous		
1928																	
May-----	13	4 2	Inches	° F	° F	° F		5, 159	4, 602	119	3	294	141		0	0	
June-----	13	8 75	8 94	310	86	6 68	9 77 8	9, 025	4, 350	450	8	909	3, 208	96	4		
July-----	12	12 4	5 25	274	91	3 70	7 81 0	{ 5, 535 43, 356 }	4, 102	256	2	865	225	{ 36, 894 23 }	62		
August-----	13	14 1	1 90	248	93	4 71	1 82 3	11, 700	7, 444	654	123	1, 573	1, 177	175	254		
September--	10	6 4	. 89	129	83	6 58	9 71 1	14, 135	8, 300	612	58	3, 430	977	175	533		
1929																	
May-----	12	15 4	2 83	362	82	2 62	1 72 2	4, 527	3, 713	129	4	383	277	13	8		
June-----	12	11 8	2 76	323	89	2 66	7 77 9	7, 415	5, 389	117	48	1, 569	219	69	4		
July-----	11	10 1	4 51	244	92	2 69	8 81 0	9, 432	4, 450	384	27	1, 298	1, 641	1, 562	70		
August-----	6	6 4	1 82	123	93	2 66	6 79 9	{ 16, 164 66, 031 }	8, 575	275	18	1, 432	{ 5, 550 56, 397 }	193	121		
September--	4	14 0	1 10	107	88	6 63	1 75 8	11, 069	3, 238	287	0	1, 082	5, 287	1, 150	25		

#### IMPOUNDED BAYOU STATIONS

1928															
May.....	3	0						10, 483	10, 233	100	0	100	50	0	0
June.....	3	0						15, 283	4, 883	450	0	1, 292	7, 667	991	0
July.....	3	0						7, 917	5, 359	583	0	800	1, 133	42	0
August.....	3	0						9, 867	6, 567	600	0	1, 183	1, 317	200	0
September..	3	0						15, 683	4, 725	1, 633	0	5, 992	2, 483	350	450
1929															
May.....	3	0						2, 525	1, 616	167	17	550	133	42	0
June.....	3	0						24, 925	11, 559	483	0	11, 400	975	508	0
July.....	2	0						{ 6, 387 514, 442 }	5, 513	238	12	612	0	{ 500, 008 12 }	0
August.....	3	0						25, 612	5, 675	433	0	975	3, 609	14, 900	50
September..	2	0						{ 9, 800 461, 000 }	5, 950	425	0	925	2, 425	{ 75 450, 050 }	0

\* Including the count from 1 collection which contained an extremely large number of 1 group of organisms

larval rates (11 plus), it is seen that there is little difference in the average plankton population per cubic centimeter of these two groups when the total plankton is considered. In the individual classes of organisms the major differences in the rates of occurrence are not constant when one year is compared with another except in the case of the Chlorophyceae, and here, by the elimination of an additional high record from the low breeding group for each year, this apparent constant difference may be eliminated (The average of 1,725 in 1928 becomes 439, and the average of 1,720 in 1929 becomes, 1,111 )

While it might appear logical to conclude that the absence of feeding by the larvae was responsible for the higher plankton rates in the nonbreeding groups of stations, it is believed that the environmental conditions prevalent in these areas were the principal cause of the large plankton population. In the impounded bayous the water is unshaded and has no aquatic vegetation to cover its surface. This condition, of course, favors more rapid growth and multiplication of the chlorophyll-bearing organisms than occurs in shaded or partly shaded areas in which most of the *Anopheles* breeding takes place hereabout. The group of observations made when *Anopheles* were temporarily absent also shows a high plankton count in each year. This may be explained by the fact that a part of the observations in this group were made at stations where the water had receded from marginal shaded areas in which larval protection had occurred and, at the time of observation, presented conditions similar to those in the cleared and impounded bayou areas previously mentioned.

#### RATE OF OCCURRENCE OF THE FOUR COMMONEST PLANKTON GENERA IN ANOPHELES BREEDING AND NONBREEDING WATERS

In comparing the records on the rate of occurrence per cubic centimeter of the more common plankton genera with the absence of and the presence in increasing numbers of *Anopheles* larvae, no definite trends were found. The data in Table 3 illustrate this condition as it occurred in four of the most common genera of the flagellates. In this table the findings are grouped into the same divisions in regard to larval findings as are used in the latter part of Table 2. The average numbers of these genera per cubic centimeter are given and also the percentage of the total flagellate population of the water that these four genera represent. The data show that these genera are present in abundance in each of the groups of observations and that they compose practically the same percentage of the total flagellate population in each group, with the exception of a low percentage in the impounded-bayou group of stations in 1929. This is caused by larger counts of less common genera and not by any lack of abundance of the genera under consideration. It is also noted that the temporary nonbreeding-station group in each year had a high flagellate population, and this may be explained by the fact that some of these areas are bodies of water having very little shade and are sometimes more or less fouled by the wallowing of animals. This type of location has been found to be particularly suitable for the development of large numbers of flagellates, particularly *Euglena* spp., which often become so abundant as to color the water green.

TABLE 2.—Average number of plankton organisms as compared to larval abundance rates of *Anopheles* at Mound, La

Year and number of observations	Larval abundance per 10-dip collection		Average number of organisms per cubic centimeter							
	Range	Rate	Total plankton	Flagellates	Ciliates	Amoeboids	Diatoms	Chlorophyceae	Cyanophyceae	Miscellaneous
1928										
15-----	(a)	0	11,547	6,354	683	0	1,873	2,530	317	90
10-----	0	0	10,927	7,302	532	25	2,174	574	205	115
16-----	1-5	2 13	{ 8,407 b 37,160 }	{ 4,500 c 5,535 }	458	17	736	2,552	{ 28,221 d 25 }	{ 119 e 304 }
13-----	6-10	7 46	8,535	6,194	271	37	929	706	94	167
9-----	11-15	13 33	6,672	4,511	328	47	1,203	355	61	0
3-----	16-20	18 67	12,592	10,625	633	17	642	567	108	183
6-----	21-25	23 50	10,129	4,154	400	146	3,712	1,367	167	108
3-----	26-30	28 30	9,275	6,650	433	0	1,284	800	0	0
1-----	31-35	33 00	3,100	2,475	375	0	200	50	0	0
1929										
13-----	(a)	0	{ 14,742 c 205,707 }	{ 6,113 d 11,028 }	352	6	3,219	1,461	{ 3,579 e 193,102 }	12
9-----	0	0	17,975	11,028	383	14	1,211	4,103	1,236	11
9-----	1-5	3 22	{ 7,819 b 48,548 }	{ 2,555 c 3,125 }	292	8	1,573	{ 3,014 d 43,945 }	361	103
8-----	6-10	7 60	5,812	3,125	87	40	2,050	266	141	25
9-----	11-15	13 00	8,888	6,458	255	14	639	1,275	192	0
1-----	16-20	19 00	2,725	1,975	0	200	550	0	0	33
3-----	21-25	24 00	5,517	4,059	92	0	558	92	683	0
2-----	26-30	28 00	2,963	1,125	63	0	537	1,000	238	575
1-----	31-35	32 00	4,100	950	25	0	325	1,925	300	0
2-----	36-40	38 00	4,538	1,238	150	100	325	1,000	2,025	0
2-----	41-45	42 00	3,400	2,838	225	0	262	75	0	0

COMPARISON OF LOW WITH HIGH BREEDING STATIONS (ONE COLLECTION OMITTED EACH YEAR)

1928										
29-----	1-10	4 55	8,465	5,260	374	26	822	1,725	56	202
22-----	11-35	19 70	8,616	5,448	406	61	1,776	707	85	133
1929										
17-----	1-10	5 30	6,875	2,824	196	24	1,800	1,720	257	54
20-----	11-45	22 80	6,289	4,195	174	27	527	891	430	45

a Impounded bayou

b Including 1 high count

c Including 2 high counts

## COMPARISON OF LARVAL RATES AND PLANKTON COUNTS

Table 2 gives a summary of the results of the organism counts for the two seasons under consideration, as compared to larval population, irrespective of environmental conditions in the breeding areas. The data are divided into three groups, viz, those made in the cleared and impounded-bayou areas where *Anopheles* was consistently absent, those made in areas where *Anopheles* was usually found but for some reason was temporarily absent; and those made in areas where *Anopheles* was present. The observations in the last group are divided into subgroups based on increasing larval population.

In addition to showing the general tendency for the nonbreeding waters to have a higher plankton content than the breeding waters, the data given in this table seem to indicate that among the breeding stations those having the greatest larval population have the lowest plankton population, this being true whether the total plankton or a major class of organisms is considered. By summarizing these records further, however, and making only two subgroups of breeding stations, viz, those with low larval rates (1-10) and those with high

larval protection may be alternately present and absent in the same location. This same process is in effect, of course, with the larger débris but to a lesser extent, as this material, especially the fallen trees and logs, tends to become lodged or anchored in place. Figure 3 shows an *Anopheles* breeding place formed by collections of débris.

Of the plants that serve as protection for *Anopheles* larvae, the most important perhaps are the filamentous algae which grow at or just below the surface film of the water (Fig 4). *Anopheles* larvae usually thrive in their presence. *Ceratophyllum*, *Potamogeton*, *Chara*, and others of the larger plants which grow in the water and parts of which come to lie at the water surface, provide protection in essentially the same manner as the algae. Floating plants, such as *Lemna*, *Heteranthera*, *Wolffia*, *Azolla*, etc., can not be considered as effective protection for *Anopheles* larvae because of the fact that



FIGURE 3 — An uncleared bayou during high water, showing collections of *Anopheles*-sheltering débris

their leaves lie on top of the water surface and therefore do not hide the larvae from their enemies in the water. Patches of such plants, however, when not too dense, usually harbor attached algae or other materials which favor mosquito breeding. Furthermore, these algae and other materials tend to keep the leaves of the floating plants from forming a compact surface mat which would mechanically inhibit the production of *Anopheles*. In the same manner, plants such as *Castalia*, *Nelumbo*, *Saururus*, grasses, etc., which root on the bottom and extend to or through the water surface, while not of themselves providing much protection, serve as attachment and lodging for other protective materials.

Plants which have large root and stem masses below the water surface and send shoots above the water, sometimes in such profusion as to hide the water surface entirely, may or may not provide good

TABLE 3—Occurrence of the four commonest flagellate genera, compared to *Anopheles* larval abundance, Mound, La., 1928 and 1929

Year and station group	Observations	Larval range	Average per 10-dip collection	Average rate per cubic centimeter						Percentage of total flagellates
				Euglena	Trachelomonas	Chlamydomonas	Phacus	Total of four genera	Total of all flagellates	
1928										
Impounded bayou.....	15	0	-----	1,440	2,127	530	123	4,220	6,353	66.42
Other nonbreeding.....	10	0	-----	3,040	1,309	592	312	5,253	7,302	71.94
Low larval density.....	a 29	1-10	4-6	1,234	1,700	336	155	3,425	5,260	65.12
High larval density.....	22	11-35	19-7	1,793	1,026	1,207	60	4,086	5,446	75.03
1929										
Impounded bayou.....	15	0	-----	1,320	935	1,345	167	3,770	6,882	54.78
Other nonbreeding.....	9	0	-----	5,097	1,328	1,281	447	8,153	11,028	73.93
Low larval density.....	a 17	1-10	5-3	522	588	922	102	2,134	2,824	75.57
High larval density.....	20	11-45	22-8	831	921	1,231	124	3,107	4,195	74.07

a 1 observation omitted, as in Table 2

#### EFFECT OF IMPOUNDING WATER ON ANOPHELES BREEDING

The fact that the waters of this locality are well stocked with the mosquito-destroying fish *Gambusia affinis* Raf makes it necessary that protection of some sort be afforded *Anopheles* larvae before development can take place. This was well illustrated by Van Dine (7), who cleared and impounded a 1-mile section of one of the bayous near Mound and thereby eliminated the production of *Anopheles* in the area (Fig 2). This section was completed in 1916, has been under observation continuously since that time, and has maintained itself mosquito free. Although other factors in addition to those of lack of vegetation and debris may be concerned in bringing about this condition in cleared and impounded areas generally, it is certain that unless protected from their enemies *Anopheles* larvae do not develop in numbers in this vicinity.

#### DISCUSSION OF FACTORS IN LARVAL PROTECTION

Protection for larvae is afforded in waters in their natural state hereabout by floating vegetable debris, and by plants which grow on or at the surface of the water in such a manner as to conceal the larvae from their enemies. Floating debris may be more or less readily divided into two classes, viz, "large" debris and "small" debris. The former consists of logs, fallen trees, sticks, leaves, etc; it does not form a particularly compact mass on the water surface and is not as effective in protecting the larvae as is the small debris. The latter is composed of small rotting particles of vegetable matter resembling very coarse sawdust which collect on the water surface and form mats of various sizes, in which the larvae are well protected from their enemies. Floating debris is much affected by wind and by the rising and falling water levels caused by alternating periods of rain and drought. In open areas and in the absence of vegetation or large debris to serve as anchorage, a wind will sweep the smaller material to shore, where a lowering water level will shortly strand it until a later rain causes the water to rise. In this manner good

algae, etc., which afforded excellent protection for larvae. A period of drought caused the marginal areas to become dry, and afterward intensive breeding occurred all over the *Castalia*-covered area.

#### PLANTS AS CULICIDES

A summary and discussion of the literature on the larvicidal effect of plants on mosquitoes has recently been published by Matheson (4). It is clear from his discussion that certain plants are definitely associated with lack of mosquito breeding in certain localities and not in others, in spite of considerable work on the subject, however, the exact factors causing these conditions are still unknown. It is impossible at the present time to ascribe to any of the plants growing hereabout definite larvicidal qualities against *Anopheles* mosquitoes, except those that are involved in limiting breeding in a purely mechanical manner, as will be shown later.

#### EFFECT OF DÉBRIS AND OF ALGAE AND OTHER PLANTS ON LARVAL ABUNDANCE RATES AND PLANKTON CONTENT OF WATER

Table 4 shows the effect on the larval abundance rates of the presence and absence of débris in the breeding areas, irrespective of other protective agents, and also the effect of the presence of filamentous algae within each of these groups. It is here shown that in the areas lacking protective material no *Anopheles* breeding occurred and that in the areas containing large débris only the larval rate was much lower than in those where both large and small débris occurred. When filamentous algae are present with large débris the effect is to increase the larval rate considerably; when they are present with large and small débris, however, the larval rate is not essentially different than it is in their absence. These findings indicate that small débris and filamentous algae afford about the same degree of protection to *Anopheles* larvae. The percentage of large or mature larvae found in larval collections from the breeding areas is given to show that protection is afforded the insects by these conditions throughout larval life.

TABLE 4—*Effect of débris and algae on larval abundance rates at Mound, La., 1928-29*

Number of observations	Protective material	Larval rate per 10 dips	Large larvae
		Number	Per cent
7	None.....	0	0
48	Large débris.....	8 6	23 3
54	Large and small débris.....	13 01	23 7
21	Large débris, no algae.....	5 4	26 9
27	Large débris, with algae.....	11 2	21 7
14	Large and small débris, no algae.....	13 9	20 8
40	Large and small débris, with algae.....	12 8	24 6

Table 5 gives additional data on the occurrence of *Anopheles* larvae with protective materials and also shows the nature of the plankton content of the water in each of the groups of breeding areas. This table shows that the larval abundance rates in the groups having large débris alone, those having grass, weeds, etc., and those having

larval breeding areas. In this class are such plants as *Jussiaea*, knotweed, smartweed, climbing hempweed, etc. *Jussiaea*, for instance, by its habit of growth seems to offer excellent protection for larvae, and, when only small quantities of it are present in an area, larvae of all sizes are likely to be found among its algae-coated stems and roots. However, when an area becomes thickly covered with this plant, even though conditions in spots appear favorable, *Anopheles* breeding is often very sparse or wanting. While this plant grows luxuriantly under a variety of conditions, it shows a particular tendency to grow rapidly and to form a dense covering over the water surface in newly cleared or in cleared and impounded areas. For



FIGURE 4—A patch of green algae mangled with debris. *Anopheles* larvae are usually abundant in such locations.

some reason this luxuriant growth has not been observed to persist as a rule in the same location for several successive years.

#### ADAPTABILITY OF LARVAE

Our common *Anopheles* mosquitoes are undoubtedly adaptable to a wide variety of breeding places, and, lacking their preferred habitat, they may readily choose another; that is to say, the fact that *Anopheles* larvae are not found in a certain location is no reason for believing that conditions in that area are such that they can not develop there. For example, in the course of airplane dusting operations a few years ago it was found that whereas the shrub and *Nelumbo* covered marginal areas in a large lake were breeding *Anopheles*, no larvae were present in a large central area of the lake which was more or less covered with growths of *Castalia*. Both areas had a thick subsurface growth of *Ceratophyllum*, *Potamogeton*, *Utricularia*,

Table 5 also shows the effect of large and small débris, alone and in combination with other protective agents, on larval rates and on the plankton content of the water. When small débris is found in association with weeds and grass or with algae the rates of larval abundance are not markedly different from those in similar groups but with only large débris present. In those groups that combine large and small débris with the algae (as in the lower part of Table 5), the group showing blue-green filamentous algae has a low larval rate. However, the group in which blue-green filamentous algae were found mixed with green filamentous algae had much higher rates than the groups that had green algae alone. This fact would seem to indicate that if there is any relation between the presence of filamentous blue-green algae and the scarcity of *Anopheles* larvae, this deterrent influence is overcome hereabout by the presence with it of green algae.

The data on plankton organisms show that each of the groups is well supplied with plankton food for the larvae, and that the groups having the highest larval rates tend to have the lowest plankton counts, as has been previously noted. It is not believed that this condition is particularly significant, except as it indicates that in this locality the most favorable breeding conditions occur in more or less shaded areas, while the chlorophyll-bearing organisms become more abundant where the water surface is open.

#### EFFECT OF CYANOPHYCEAE ON LARVAL ABUNDANCE

Table 5 shows that filamentous blue-green algae, when unassociated with green filamentous algae in the breeding areas, apparently limits the production of *Anopheles*. This might readily be ascribed to the fact that the growth habit of the filamentous Cyanophyceae is such as to give very little protection to the larvae, since the patches of blue-green algae when found alone are usually small and are easily blown about by the wind, or when growing attached to débris they usually form only a very narrow fringe. However, Boyd (2) reported a negative relation existing between the unicellular Cyanophyceae and anopheline larvae, and recently Allison and Morris (1) have shown that blue-green algae possess the power of nitrogen fixation. In studying some factors in mosquito ecology, Senior-White (6) reached the conclusion that "saline ammonia is inhibitory to *Anopheles* breeding, save in the case of the *rossi* group, in amounts exceeding one part per million." It may be, therefore, that in the areas hereabout where large quantities of Cyanophyceae are present, the water is given a saline ammonium content sufficient to make it unfavorable for *Anopheles* production.

#### EFFECT OF LEMNA ON LARVAL ABUNDANCE

The effect of the presence of *Lemna* spp. in various quantities on larval abundance and on the plankton content of the water is shown in Table 6. In this table the observations on the abundance of this material have been divided into four groups, viz, those without *Lemna* (0), those with only scattered patches (+), those with an abundance but not sufficient to give the water a continuous surface mat(++), and those with a complete surface mat of the plant(+++). It is noted that the presence of considerable quantities of this material

filamentous blue-green algae with and without weeds and grass are somewhat similar for both the years under consideration. In the groups with filamentous green algae alone, and those with a mixture of the green and blue-green material, the figures for the two years are not at all alike. In 1928 the presence of blue-green algae appears to have had a somewhat inhibitive effect on *Anopheles* breeding, as the stations having this material alone gave the lowest larval rate of any group in which filamentous algae were present, while those with green algae alone had a very high rate, and those with a mixture of the two forms had an abundance rate indeterminate between the two. In 1929, however, the effect of the presence of blue-green algae is not so clear, because, while the group of stations in which this material was found alone gave a lower rate than that in which green algae appeared alone, as in 1928, the group having a mixture of the two forms had the highest abundance rate. Large debris alone does not appear to afford very effective larval protection, but weeds, grass, etc., do. The presence of visible patches of filamentous green algae with large debris greatly increased the larval rate, either when found alone or when mixed with blue-green algae. When no algae other than blue-green was observed the larval density was much lower than that of any group in which algae occurred in association with debris.

TABLE 5—Effect of debris and other materials on *Anopheles* abundance rates and plankton content of the water, Mound, La

WITH LARGE DEBRIS ONLY

Year and observation	Larvae per 10-dip collection	Visible filamentous algae		Grass, weeds, etc.	Organisms per cubic centimeter							
		Green	Blue-green		Total plankton	Flagellates	Ciliates	Amoeboids	Diatoms	Chlorophyceae	Cyanophyceae	Miscellaneous
1928												
9.....	2.6	—	—	—	5,624	4,072	417	50	555	269	11	250
6.....	10.2	—	—	+	5,820	3,917	325	4	1,425	154	4	0
2.....	4.0	—	+	±	30,350	9,212	125	213	1,350	18,237	250	963
6 <sup>a</sup> .....	7.8	+	+	±	13,708	8,221	625	125	2,746	1,446	325	220
7.....	18.1	+	—	±	8,400	4,125	354	18	2,357	1,180	193	161
1929												
2 <sup>a</sup> .....	0	—	—	—	18,225	12,688	350	0	150	27	5,000	0
3.....	8.0	—	—	+	8,808	7,350	225	8	717	467	8	33
2.....	6.0	—	+	±	6,050	2,750	163	13	2,575	387	25	137
3.....	16.0	+	+	±	4,625	825	100	67	275	1,867	1,483	8
6.....	9.9	+	—	±	16,676	6,058	292	50	2,196	7,292	692	96

WITH LARGE AND SMALL DEBRIS

1928												
3.....	14.2	—	—	—	6,260	1,640	200	5	940	455	20	0
3.....	10.3	—	—	+	12,925	11,225	675	33	492	407	33	0
0.....	—	—	+	±	—	—	—	—	—	—	—	—
3.....	17.5	+	+	±	5,759	4,142	217	0	800	400	75	125
17.....	8.6	+	—	±	7,223	5,546	377	26	677	418	48	131
1929												
6.....	14.0	—	—	+	7,977	2,533	417	17	844	3,812	312	42
0.....	—	—	—	—	—	—	—	—	—	—	—	—
2.....	2.0	—	+	±	10,326	5,612	513	0	2,063	2,075	63	0
9.....	22.0	+	+	±	4,707	2,872	117	3	928	364	355	68
9.....	13.1	+	—	±	6,055	4,958	153	31	858	47	5	3

<sup>a</sup> 1 observation omitted

<sup>b</sup> Plus-minus signs indicate that some of the dips of the 10-dip collection contained grass, weeds, etc., while in other dips this material was absent

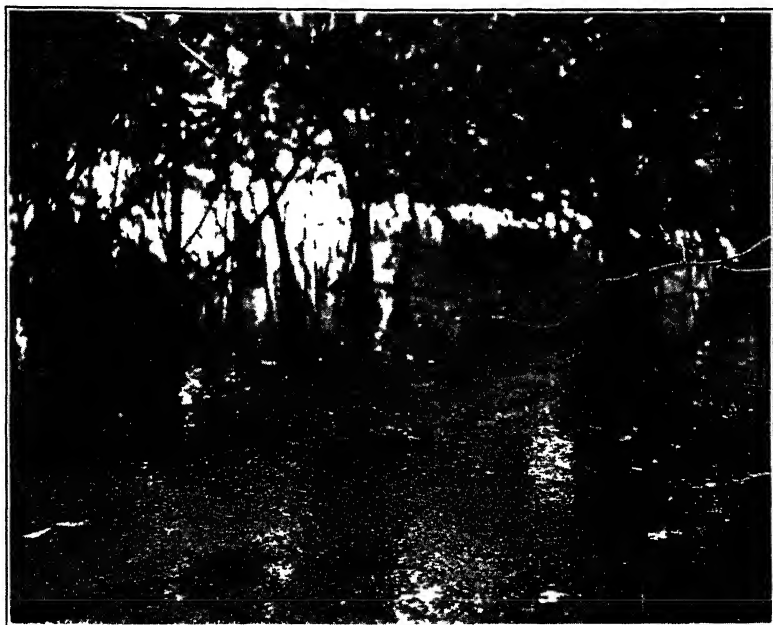


FIGURE 5—*Lemna* spp in abundance on water surface, a good environment for *Anopheles*



FIGURE 6—A small lake with the water surface entirely covered by *Lemna* spp. *Anopheles* larvae are exceedingly scarce or entirely absent in such places

(Table 6 (++) , fig 5) usually indicates good breeding conditions for Anopheles, while a complete surface mat (Table 6 (+++) , fig 6) of Lemna effectively checks breeding. It would also appear from the records of 1928 that even small quantities of Lemna (Table 6 (+)) tend to increase larval rates. This condition was reversed in 1929, however, and it is believed that in general the quantity of Lemna present in locations of this type has no particular effect on larval protection and abundance. That larvae reach maturity in all these environments except that having a complete surface mat of Lemna is shown by the percentages of large, or approximately mature, larvae occurring in the collections.

TABLE 6—*Effect of Lemna on larval rates and on plankton counts per cubic centimeter in Anopheles breeding areas at Mound, La*

Year and number of observations	Larvae in 10-dip collections	Large larvae	Quantity of Lemna	Organisms per cubic centimeter							
				Total plankton	Flagellates	Ciliates	Amoeboids	Diatoms	Chlorophyceae	Cyanophyceae	Miscellaneous
1928		Per cent									
36-----	8 17	29 3	0	23,970	6,867	434	17	1,437	1,684	13,425	106
17-----	11 35	28 5	+	7,362	4,683	386	95	1,086	768	67	277
6-----	15 67	30 8	++	7,958	3,546	629	21	2,541	946	75	200
3-----	33	0	+++	1,875	1,175	108	0	325	200	0	67
1929											
25-----	12 72	21 7	0	9,030	4,950	235	4	801	2,304	721	15
18-----	7 22	17 7	+	31,977	5,777	240	42	1,725	24,066	76	51
4-----	25 75	6 8	++	4,550	1,244	87	50	469	1,381	1,175	144
0-----			+++								

Lemna in large quantities causes lack of activity among chlorophyll-bearing plankton by cutting off most of the sunlight from the water surface. This is illustrated by the figures in plankton density given in Table 6. As usual, the highest plankton counts are associated with the lowest larval rates except in the case of the group in which a complete mat of Lemna covers the water surface; here, on account of the absence of sunlight, the plankton count is low and, for mechanical reasons, Anopheles larvae are exceedingly scarce. If the observations on the occurrence of Anopheles larvae with Lemna are separated into groups on the basis of those made at stations having large debris in addition to Lemna and those made at stations having both large and small debris in addition to Lemna, it will be found that the same general conclusions with respect to abundance of Anopheles in the presence of increasing quantities of Lemna apply.

#### HYDROGEN-ION CONCENTRATION OF WATER IN RELATION TO PRESENCE OF ANOPHELES LARVAE

At the time of each observation on larval abundance a hydrogen-ion determination of the water was made by the use of a LaMotte colorimetric set. Table 7 gives a record of these determinations, which are divided into three parts—those made in the impounded areas, those made in areas from which Anopheles was temporarily absent, and those made in areas in which Anopheles was found. The data show that the waters in this locality do not have a particularly wide pH range and that they are predominantly alkaline in reaction in each

condition of the organism in the larval gut and consequently the chance of its being recognized. In watching, under the microscope, larvae in the act of feeding, no selection of food particles was observed, the larvae ingesting whatever was presented, provided it was of suitable size. The larvae feed upon filamentous algae in two ways. Sometimes they ingest the entire filament and sometimes they run a filament between the mandibles, chewing and sucking out the cell contents as the filament goes through the mouth, and then discard the empty filament. It is of course impossible to identify any material subjected to this latter method of treatment. When the entire filament is ingested, however, a sufficient number of cells remain intact to make identification possible.

TABLE 8—*Organisms of Anopheles breeding areas, and those found in larval dissections, Mound, La*

Organism	Number of times encountered			Organism	Number of times encountered		
	In water	In gut and in water	In gut only		In water	In gut and in water	In gut only
<b>Cyanophyceae</b>				<b>Chlorophyceae—Continued</b>			
Anabaena.....	16	3	0	Oedogonium.....	29	15	12
Lyngbya.....	8	1	0	Spirogyra.....	26	8	0
Nostoc.....	7	2	0	Tribonema.....	10	1	1
Oscillaria.....	12	7	1	Ulothrix.....	6	1	1
Chroococcus.....	3	1	0	Vaucheria.....	5	1	0
Merismopodium.....	3	0	1	Others.....	30	11	0
Synechocystis.....	0	0	1	<b>Diatoms</b>			
Others.....	3	0	0	Unicellular.....	63	53	0
<b>Chlorophyceae</b>				Filamentous.....	11	4	1
Actinastrum.....	3	0	0	<b>Protozoa</b>			
Ankistrodesmus.....	1	0	0	Amoeboid—			
Arthrodesmus.....	1	0	0	Arcella.....	19	10	20
Characium.....	2	1	1	Diffugia.....	8	1	3
Chlorobotrys.....	0	0	1	<b>Flagellata—</b>			
Chlorococcus.....	2	0	1	Ceratium.....	2	0	0
Chlorella.....	2	1	0	Chilomonas.....	14	1	0
Closterium.....	12	4	4	Chlamydomonas.....	45	26	1
Coelastrum.....	3	1	1	Cryptomonas.....	1	0	0
Cosmarium.....	5	3	2	Dinobryon.....	2	0	0
Crucigenia.....	9	2	1	Euglena.....	60	45	0
Dictyosphaerium.....	1	1	0	Eudorina.....	14	1	1
Euastrum.....	1	1	0	Glenodinium.....	18	1	0
Gleocystis.....	2	0	0	Gonium.....	9	2	0
Kirchneriella.....	1	1	0	Mallomonas.....	1	0	0
Ophioecium.....	12	0	0	Notosolenus.....	1	0	0
Oocystis.....	0	0	1	Pandorina.....	16	2	2
Pediastrum.....	12	2	0	Phacus.....	38	21	2
Planktothraera.....	1	0	0	Platydorina.....	4	1	0
Scenedesmus.....	16	3	1	Pleodorina.....	2	1	0
Saurastrum.....	1	0	1	Synura.....	11	1	0
Tetrasira.....	16	0	0	Trachelomonas.....	62	39	0
Tetraspora.....	2	0	0	Urocellopsis.....	6	1	0
Xanthidium.....	1	0	0	Uroglana.....	1	1	0
Cladophora.....	2	0	0	Others.....	50	7	1
Hormidium.....	1	0	0	Pollen.....	7	4	5
Mougeotia.....	8	1	1	Spores.....	13	11	22

It is believed that the difference in the character of food materials rather than the exercise of any preference on the part of the larvae accounts for the fact that some available organisms are recognized in the gut in a larger percentage of cases than others which are apparently as readily available for food. Hard-shelled organisms such as diatoms and certain protozoans are not easily crushed and are nearly always found in gut examinations when they are present in the water, while soft-bodied ciliates and the rarer flagellates are seldom recog-

group of stations. Most of the pH values in the impounded areas are slightly higher than in the other groups, the mean being 8.01, while the mean in the breeding areas is 7.42. Undoubtedly the higher pH value in the impounded areas is connected with the higher plankton content of these areas, as it is known that the photosynthetic action of the algae decreases the hydrogen-ion concentration of waters in which they occur. In Table 7 the readings have been divided, for each group of observations, into those made in the morning and those made in the afternoon. The afternoon readings in each group have a somewhat higher range than the morning readings, illustrating the diurnal variation in hydrogen-ion concentration discussed by Matheson and Hinman (5). The data give no indication that the hydrogen-ion concentration is essentially different in the breeding and non-breeding waters. The five highest readings in the breeding areas were associated with high larval rates (from 14 to 37 per 10 dips). Except for these, there was little or no correlation between larval abundance and either high or low readings.

TABLE 7.—*Hydrogen-ion concentration of water areas at Mound, La., 1928 and 1929*

Station	Time of day	Number of observations in which the pH readings came within the indicated ranges of hydrogen-ion concentration													
		6.8-6.9	7.0	7.1-7.2	7.3-7.4	7.5-7.6	7.7-7.8	7.9-8.0	8.1-8.2	8.3-8.4	8.5-8.6	8.7-8.8	8.9-9.0	9.1-9.2	
Impounded bayous	Morning	1		1		4	4	2	2	1			3		
	Afternoon					1	2	4	3					2	
Other nonbreeding areas	Morning	0	2	5	2	2	1			1		1			
	Afternoon		1					3	1						
Breeding areas	Morning	8	10	8	12	9	8	1	1			1			
	Afternoon	3	0	6	9	7	4	1	1		1	1		2	

## RESULTS OF LARVAL DISSECTION STUDIES

Dissections were made of 31 lots of larvae in 1928 and of 32 lots in 1929 (a total of 272 larvae) to ascertain the nature of the food ingested. The larvae were not killed at the time of collection but were placed in water from the collecting area, brought immediately to the laboratory and examined. In making these dissections a larva was placed in clear water, the head and the last two or three abdominal segments were removed, and then by the use of needles and forceps the gut was drawn out, placed in a drop of clear water on a slide, and the contents squeezed out. A cover glass was then applied and the mount examined. A summary of the results of these dissections is given in Table 8.

This table shows the number of times each genus of organism was found in the water from the collecting areas, the number of times the genus was represented in the gut contents of the larvae examined from these locations, and also the number of times the genus was found in the larvae when not observed in the water sample. It is seen from these records that most of the genera, whether commonly or rarely present in the breeding areas, are liable to ingestion by the larvae. Whether this is by choice or chance is problematical. The amount of maceration undergone by an organism in the mouth of a larva and its degree of digestibility undoubtedly influence greatly the

in composition between the two groups were found, nor were any consistent differences observed between the breeding waters having high and those having low larval rates. Data are given for the flagellate Protozoa, showing that the four commonest genera within this class compose approximately the same percentage of the total flagellates in each group of waters.

The range of hydrogen-ion concentration in the breeding and non-breeding waters was found to be essentially the same, and all groups of waters were principally alkaline in reaction. The mean of the readings in an unshaded impounded bayou, however, was higher than that in the breeding areas (8.01 as against 7.42).

Examinations of the gut contents of larvae showed that all organisms of suitable size when present in the water are likely to be ingested, but that some available forms are present in the gut less often than others. This may have resulted from the fact that the preferred habitat of these organisms does not coincide with that in which larvae usually feed, or it may have been that the amount of maceration which the softer-bodied organisms undergo greatly reduced their chances of being recognized.

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nized. Another factor to consider in discussing these results is that it is not definitely known just what organisms are most likely to be drawn into the current set up by the mouth parts of the larvae. Some organisms doubtless come within larval range more frequently than others because their favored stratum of existence is at or close to the surface film in which *Anopheles* larvae do most of their feeding. As the water sample from which plankton examinations were made included the top half inch of water, organisms are probably included which may usually be outside of the larval feeding range.

It is noticeable that certain organisms, notably amoeboid Protozoa, the green alga, *Oedogonium*, and spores, frequently appear in the gut when not found in the water examination. It is probable that these organisms were lacking in the water sample as a result of the method of sampling. In order to obtain water free of trash the samples were drawn from clear surface areas sometimes distant a few inches from the place where the larvae were feeding. Had the water examination included a more intensive study of the adherents of the protective materials these discrepancies might not have been so evident.

#### SUMMARY

Observations were made on various environmental factors prevailing in *Anopheles* breeding and nonbreeding areas in order to determine if possible the factors which influence the increase or decrease of larval abundance.

Protection for larvae is necessary in the water areas studied on account of the presence in abundance of *Gambusia affinis*, a mosquito-destroying fish, and other natural enemies. Protection is provided in the breeding areas by floating vegetable debris and by various species of plants. The best protection is afforded by small floating debris and by filamentous algae. Water areas containing filamentous blue-green algae in the absence of filamentous green algae were not found to be very favorable for larvae. *Lemna* affords some protection to the larvae, but when it occurs in such profusion as to form a complete mat over the water surface it almost completely inhibits the breeding of *Anopheles*.

Larval abundance, as measured by the number of larvae occurring in collections of 10 dips of surface water, was not greatly affected by variations in the mean summer air temperatures, which in each month were above 70° F.

Under certain conditions a rapid decrease in surface water was found to reduce the number of larvae, as a result of the stranding of protective material.

The plankton content of the breeding and nonbreeding waters was computed on the basis of the occurrence of the various groups of organisms per cubic centimeter of surface water. It was found that as a rule larger numbers of plankton organisms occurred in the nonbreeding waters. This was explained in part by the fact that the breeding waters usually are more shaded and the surface is covered to a greater extent by vegetation and debris, and this condition results in the development of fewer chlorophyll-bearing organisms. The character of the plankton as regards composition by classes of organisms in the various groups of waters in which *Anopheles* breeds or does not breed was found to vary considerably, but no consistent variations

# LIFE HISTORY OF THE RABBIT STOMACH WORM, *OBELISCOIDES CUNICULI*<sup>1</sup>

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## INTRODUCTION

In 1923 Graybill (8)<sup>2</sup> described from domestic rabbits (*Oryctolagus cuniculus*) in the United States a new genus and species of stomach worm which he called *Obeliscus cuniculi*. The following year Graybill (9) noted that the generic name *Obeliscus* was preoccupied and proposed the name *Obeliscoides*, type *Obeliscoides cuniculi*, to replace *Obeliscus* Graybill, 1923. Morphologically *Obeliscoides cuniculi* is related to *Graphidium strigosum* (Dujardin, 1845), a stomach worm occurring in wild and domestic rabbits in Europe. In common with the latter, *Obeliscoides cuniculi* may be visibly injurious to its host. Schwartz and Shook (18) have noted that the European stomach worm of rabbits is known to produce disturbances of various sorts that affect the health of rabbits and that the American stomach worm of rabbits has been found to produce ulceration of the stomach wall.

Specimens of *Obeliscoides cuniculi* from domestic and wild rabbits have been received in the Zoological Division of the Bureau of Animal Industry from ten States, namely, Florida, Iowa, Kansas, Louisiana, Maryland, Nebraska, New York, Ohio, Texas, and West Virginia, and from the District of Columbia. It is evident, therefore, that this parasite is widely distributed in this country.

The morphological features of the adults of *Obeliscoides cuniculi* have been described by Graybill (8) and Chandler (3), but no information is given in their reports concerning the preparasitic development of these worms and of the immature stages within the host. Since information concerning the life history of a parasite is essential as a basis for rational control measures, it is important that the basic facts in the life history of *Obeliscoides cuniculi*, particularly those relating to its free-living stages, be ascertained. The investigation described in this paper was undertaken principally for the purpose of discovering facts in the preparasitic development of *Obeliscoides* that might lead to practical methods of controlling this parasite in rabbitries. This problem was suggested to the writer by Benjamin Schwartz, of the Zoological Division of the Bureau of Animal Industry, and was carried out under his direction and supervision.

## METHOD OF INVESTIGATION

Eggs of *Obeliscoides cuniculi*, obtained from several females, were transferred to small glass jars containing a mixture of fresh, sterile rabbit feces and animal charcoal. After the eggs had incubated for

<sup>1</sup> Received for publication Aug. 7, 1931, issued April, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 418.



from  $18\mu$  to  $22\mu$  in maximum width, indicating considerable growth during this stage of development. During the second stage, as well as during the first, the larvae feed most of the time, and as development progresses the wall of the intestine becomes darker. Before the second lethargus is completed the opening of the pharynx closes, as shown in Figure 3, B.



FIGURE 3—A, Second-stage larva of *Obeliscoides cuniculi*, B, anterior end of a late second-stage larva showing the mouth closed.

#### INFECTIVE LARVAE

In 6-day-old cultures the larvae present were usually in the third stage, that is, they were in the ensheathed infective stage. In some cases, particularly when larvae had been kept in water for several days, the sheaths were absent because of their having been cast off.

Infective larvae appear less granular than preinfective larvae (Fig 4, A). The former are from  $653\mu$  to  $710\mu$  long and about  $22\mu$  in maximum width. The maximum length of the infective larvae is somewhat less than that attained by the second-stage larvae. This is due to the fact that the tail of the infective larva is smaller than that of the second-stage larva. Several striking structural changes are apparent in this stage. The mouth is closed, and the constriction at the base of the lips has disappeared. The pharynx is no longer present, and

there is a slight opening at the beginning of the esophagus. (Fig 4, B.) The esophageal bulb has become more slender, and the valve is no longer present. The esophagus is from  $167\mu$  to  $174\mu$  long and is about one-twelfth the body length. The excretory pore is clearly visible and is located just below the nerve ring, approximately  $110\mu$  to  $125\mu$  from the anterior end of the body. The nerve ring appears as a light band just above the excretory pore, situated about

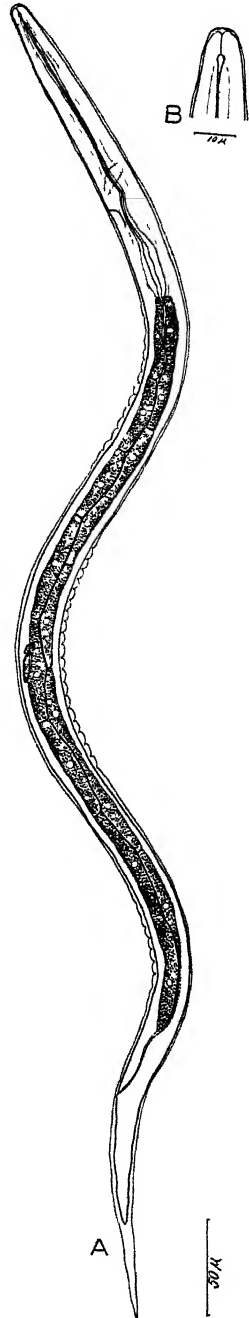


FIGURE 4—A, Third stage larva of *Obeliscoides cuniculi*, B, anterior end of the third-stage larva showing the absence of the pharynx.

about 10 days at room temperature ( $20^{\circ}$  to  $24^{\circ}$  C), the infective larvae were recovered by means of the Baermann apparatus. Pre-infective larvae were recovered from these cultures by transferring a small quantity of the culture material to glass dishes containing water and isolating the larvae with the aid of a microscope.

In order to study the course of development of the pre-infective larvae, *Obeliscoides* eggs were cultured in water. This method, however, was not suitable for the study of larval development, since the majority of the developing larvae disintegrated before reaching the infective stage. In one instance, however, the entire parasitic development took place in a water culture.

#### DESCRIPTION AND DEVELOPMENT OF THE EGGS

The eggs of *Obeliscoides cuniculi* are usually elliptical in shape and are provided with two thin membranes. In a series of measurements involving about 100 eggs the variation in length was from  $75\mu$  to  $91\mu$ , and the variation in width was from  $42\mu$  to  $53\mu$ . Graybill (8) states that the eggs are  $76\mu$  to  $86\mu$  long by  $44\mu$  to  $45\mu$  wide, whereas Chandler (3) reports a somewhat greater range in size, namely,  $80\mu$  to  $92\mu$  in length by  $56\mu$  to  $64\mu$  in width. Segmenting eggs as small as  $60\mu$  by  $38\mu$  and as large as  $152\mu$  by  $45\mu$  have been found occasionally, but such extreme sizes are rare and possibly such eggs are abnormalities.

Eggs found in fresh rabbit feces, which were examined a few minutes after they were passed, were in about the 32-cell stage, as shown in Figure 1. In tap water the eggs hatched in about 30 hours.

#### DESCRIPTION AND DEVELOPMENT OF THE LARVAE

##### PREINFECTION LARVAE

The first-stage larvae, shown in Figure 2, are characteristically rhabditiform and resemble the first-stage larvae of related strongyles. The newly hatched larvae are from  $320\mu$  to  $330\mu$  long; they increase in size gradually during the first stage. The principal measurements of these larvae, made at various times after hatching and while they were still in this stage, are as follows. Length,  $375\mu$  to  $448\mu$ , maximum width,  $18\mu$ ; length of esophagus,  $85\mu$  to  $115\mu$ ; length of tail,  $65\mu$  to  $83\mu$ . These measurements indicate that the first-stage larva grows considerably from the time that it has hatched until it is ready to molt.

The span of life of the first-stage larvae is comparatively brief, second-stage larvae were observed about 65 hours after hatching. The latter, as shown in Figure 3, A, differ but slightly from those of the previous stage. The outstanding difference is the larger size of the second-stage larvae. They range from  $471\mu$  to  $750\mu$  in length and



FIGURE 1—  
Egg of *Obeliscoides cuniculi* from fresh rabbit feces

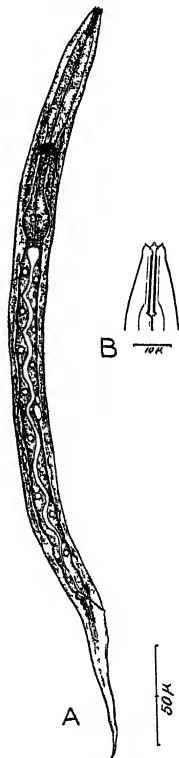


FIGURE 2—A, First-stage larva of *Obeliscoides cuniculi*, B, anterior end of the first-stage larva showing lips and pharynx

the water had remained on the skin for a short time it was transferred to a glass slide by means of a pipette. Practically all the larvae that were originally placed on the skin were recovered and were found to have retracted within their sheaths, after several minutes some of them became active. The area of the skin exposed to the larvae appeared normal. In another experiment, about 2,000 infective larvae were put on the skin of each of two rabbits, from which the hair had been clipped. One rabbit was killed eight days later and no larvae were recovered from the lungs and stomach. Examination of the lungs showed no petechial hemorrhages or other lesions indicative of infestation with nematode larvae. The second rabbit was killed 16 days after it had been exposed to a cutaneous infection. no worms were recovered from the lungs and stomach.

Another experiment was conducted in accordance with the technic described by Goodey (4). The skin of a 2-day-old rat was stretched, hair upward, on a cork ring, floated in a beaker containing warm physiological salt solution, and kept in an incubator at a temperature of 37° C. A small drop of water containing about 100 larvae was placed on the piece of skin and allowed to evaporate in the incubator. Two hours after the larvae had been placed on the skin and about 90 minutes after the drop containing larvae had evaporated, a drop of water was placed on the rat skin and then removed to a slide by means of a pipette. A microscopic examination revealed many larvae, they were still ensheathed. No larvae were found in the salt solution.

The rat skin was then fixed in 70 per cent alcohol, and the superficial layers were mechanically separated from the deeper layers. These layers were then cleared in an alcohol-phenol mixture. Several ensheathed larvae were found on the surface of the skin, but no larvae were found in the subcutaneous layers. This experiment was repeated by using the skin of a 3-day-old rat, and similar results were obtained.

These observations indicate that infection does not take place through the skin. Skin penetrators, such as the larvae of various species of hookworms, penetrate rat skin under the experimental conditions described above.

#### REACTION TO COLD

Nematode larvae vary considerably in their ability to withstand low temperatures. Cameron (2) reports that infective larvae of *Monodontus trigonocephalus* did not revive after being frozen for a few minutes. According to Ransom (15), the infective larvae of *Haemonchus contortus* are very resistant to cold. He found that after larvae in sheep feces had been kept outdoors at temperatures ranging from 21.6° to -13.8° C for 85 days they were still alive. Schwartz (16) reports that infective larvae of *Bustomum phlebotomum* (= *Monodontus phlebotomus*) which were frozen solid for about 15 hours, when thawed again became active. Schwartz and Price (17) found that the infective larvae of *Stephanurus dentatus* can withstand a temperature of -19° C for six hours but are killed when exposed to this temperature for nine hours. Monnig (12) found that the infective larvae of *Trichostrongylus* spp. from sheep were still alive after an exposure of 14 days to 0° C. Orllepp (13) reported that the

95 $\mu$  to 102 $\mu$  from the anterior end of the body, and encircling the esophagus obliquely. The genital primordium is located about 349 $\mu$  to 400 $\mu$  from the anterior extremity.

Table 1 shows the principal measurements of the first, second, and third stage larvae. These data show that the larvae grow considerably in the first stage and continue to grow in the second stage, at which time they attain a maximum length of 750 $\mu$ , which is nearly two and one-half times the size of the newly hatched larvae. In the third stage the larvae neither grow nor develop beyond the stage which they had attained after the second molt.

TABLE 1—Principal measurements (microns) of 10 *Obeliscoides cuniculi* pre-parasitic larvae of each stage

Items	FIRST STAGE									
	Measurements of larva No									
	1	2	3	4	5	6	7	8	9	10
Length of body.....	375	375	382	383	395	402	418	440	440	448
Maximum width of body.....	18	18	18	18	18	18	18	18	18	18
Length of pharynx.....	15	15	15	15	15	15	15	15	15	15
Length of esophagus.....	85	85	95	95	101	110	110	102	115	115
Distance of nerve ring from the anterior extremity.....	75	75	72	72	80	77	84	87	82	82
Distance of excretory pore from the anterior extremity.....									110	
Distance between genital primordium and the anterior extremity.....	203	203	205	205	223	215	213	216	218	228
Length of tail.....	65	65	71	68	76	76	83	80	79	83

SECOND STAGE										
Length of body.....	471	524	570	577	585	590	615	669	722	750
Maximum width of body.....	18	18	22	22	22	22	22	22	22	22
Length of pharynx.....	15	15	15	15	15	15	15	11	11	11
Length of esophagus.....	118	120	120	120	120	120	120	134	137	157
Distance of nerve ring from the anterior extremity.....	86	86	91	91	91	91	98	95	95	98
Distance of excretory pore from the anterior extremity.....	98	98	102	102		114	114	114	118	
Distance between genital primordium and the anterior extremity.....	243	273	296	296	304	293	304	335	380	
Length of tail.....	84	76	95	98	98	102	114	95	98	102

THIRD STAGE										
Length of body.....	653	662	665	686	686	690	702	702	710	710
Maximum width of body.....	22	22	22	22	22	22	22	22	22	22
Length of esophagus.....	167	171	174	167	167	174	171	171	174	174
Distance of nerve ring from the anterior extremity.....	95	99	95	102	95	95	98	102	98	102
Distance of excretory pore from the anterior extremity.....	110	121	110	129	110	110	117	125	114	125
Distance between genital primordium and the anterior extremity.....	349	361	357	361		387	364	387	387	400
Length of tail.....	65	57	65	57	61	65	62	57	68	61

## EXPERIMENTS WITH INFECTIVE LARVAE

### ATTEMPTS TO INDUCE SKIN PENETRATION

A small drop of water containing about 100 larvae was placed on a portion of the skin of a young rabbit, from which the hair had been clipped. The rabbit was kept under restraint until the water evaporated. Two hours later a few drops of distilled water were placed on the area of skin that had been exposed to the larvae, and after

larvae were kept at a freezing temperature for about 29 per cent of the time during which they were exposed to outdoor conditions. At the end of this period the jars were removed to the laboratory and kept at room temperature for one day, a large number of active larvae were recovered from each jar by means of the Baermann apparatus.

#### REACTION TO HEAT

Like the infective larvae of other strongyles, those of *Obeliscoides cuniculi* become very active when gradually warmed. Thus, if the end of a heated glass rod is brought in contact with the underside of a glass slide containing larvae, the larvae become active and orient themselves toward the source of heat.

The view expressed by Khalil (10) that only skin penetrators are positively thermotropic is untenable, as shown by observations recorded by various helminthologists. The larvae of *Monodontus trigonocephalus*, *M. phlebotomus*, and *Trichostrongylus* spp., as determined by Cameron (2), Schwartz (16), and Monnig (12), respectively, are positively thermotropic, and the available evidence indicates that these larvae do not penetrate the skin of their hosts. The writer's observation concerning the heat reaction of *Obeliscoides cuniculi* lends additional support to the view that there is no necessary relation between the positive thermotropism of larvae and their ability to penetrate the skin of rabbits or other animals.

The effect of heat on the larvae of *Obeliscoides* was not considered to be of sufficient practical significance to warrant a study of their reaction to high temperatures.

#### REACTION TO DRYING

The infective larvae of strongyles vary greatly in their ability to resist desiccation. Looss (11) reported that infective larvae of *Strongylus* spp. and *Cylicostomum* spp. can resist desiccation in a Petri dish for 14 days, and Raffensperger (14) noted that 10 per cent of *Strongylus* spp. larvae withstood desiccation in an incubator at 26° C. for a period of four months. Ransom (15) found that *Haemonchus contortus* larvae which had been dried in feces for 35 days revived after being moistened. Ortlepp (13) reported that infective larvae of *Triodontophorus tenuicollis* revived after they had been dried in an incubator overnight at 26° C. In contrast to these observations, Looss (11) has pointed out that larvae of *Ancylostoma duodenale* perish as soon as their surroundings become dry, and Goodey (5) has found that larvae of *Necator americanus* fail to revive after they have been dried a few minutes.

The following experiments were conducted to determine the resistance of *Obeliscoides* larvae to drying.

A small drop of water containing a number of infective larvae was placed on each of several glass slides. At the moment that the water appeared to have evaporated the time was noted, and the slides remained exposed at room temperature for various periods, as shown in Table 3. At the expiration of the desired lapse of time a few drops of water were added to the dried larvae and the preparations were examined at various intervals for about 24 hours. The results of these observations are given in Table 3.

infective larvae of *Trichostrongylus tenuicollis* were able to withstand freezing when kept in an ice chest overnight. De Bleeck and Baudet (1) pointed out that the infective larvae of the intestinal nematodes of the horse, *Strongylus vulgaris*, *S. edentatus*, and *Cylicostomum* spp., withstood a temperature of 0° C for 15 days in cultures of water and feces. These writers also found that these larvae in feces survived after a 2-hour exposure to temperatures ranging from -15° to -20° C. When the larvae were placed in water and exposed again to the same temperature for six hours they remained alive. Raffensperger (14) exposed horse manure containing strongyle larvae of various species to Montana weather conditions for 20 months, some larvae did not succumb despite the fact that in the course of the experiment the temperature ranged from -18.3° to -38° C for a period of 26 days in January and February, 1929.

The effects of various low temperatures on the infective larvae of *Obeliscoides cuniculi* are summarized in Table 2. Each record is based on observations involving about 400 infective larvae. The larvae were put in glass tubes containing moist animal charcoal, and the tubes were placed in a refrigerator and removed from time to time for examination. Before the larvae were examined microscopically the tubes were kept at room temperature for about two hours.

TABLE 2—Effects of low temperatures on the infective larvae of *Obeliscoides cuniculi*, each culture involving about 400 larvae

Culture No	Period of refrigeration	Temperature of refrigerator	Results
	Hours	°C	
1	5	0	Larvae active
2	10	0	Do
3	20	0	Do
4	48	0	Do
5	168	5 to 0	Most larvae active, a few dead
6	168	0 to -4	Do
7	360	0 to -4	Do
8	720	2 to -4	Do
9	24	-18	About 20 larvae active, all the others dead
10	72	-18	About a dozen larvae active, all the others dead

In this experiment the *Obeliscoides* larvae were resistant to a temperature of from 2° to -4° C for 720 hours. The temperature during this period ranged as follows. -4°, 240 hours, -3°, 24 hours, -2° to -1°, 24 hours, 0°, 384 hours, 1°, 24 hours, 2°, 24 hours. The vitality of most of the larvae kept at a temperature of -18° for three consecutive days was destroyed.

The resistance of the larvae to winter temperatures prevailing in the District of Columbia was tested as follows. About 1,000 or more infective larvae were placed in a jar containing sterile moist sand, and an equal number of larvae were placed in a jar containing sterile and slightly moist rabbit feces. These jars were kept outdoors for 30 days, from 11 a. m., December 15, 1930, to 3 p. m., January 14, 1931. The maximum temperature during this period was 13.9° C, and the minimum temperature was -9.4°. The total time during which the temperature was -11° or lower was 211 hours, so the

larvae The larvae remained active while in contact with the stain. They were kept under observation for an hour after the stain had run in under the cover slip, but no evidence of exsheathing was observed. The stain readily penetrated the sheath, but the tissues of the larvae remained unstained. The larvae continued active until the fluid evaporated. In one case larvae were kept in a vial with the stain for 18 hours, and at the end of that time all were active and sheathed.

In their failure to exsheath when in contact with a nonpoisonous stain, the infective larvae of *Obeliscoides cuniculi* resemble larvae of other nonskin-penetrating nematodes, such as *Monodontus phlebotomus* (16), *M. trigonocephalus* (2), *Trichostrongylus* spp. (12), *Hyostrongylus rubidus* (6), and *Uncinaria stenocephala* (= *Dochmoides stenocephala*) (7). The infective larvae of *Necator americanus* (5) and *Ancylostoma duodenale* (= *Agchylostoma duodenale*) (11) differ from the above-mentioned forms in that they exsheath when in contact with a solution of an aniline stain.

Cameron (2, p. 212), who worked with aniline stain, states that "all skin penetrators which have been tested have exsheathed, while the nonskin-penetrators have not exsheathed but have been quickly killed." Although there is still a possibility that all skin penetrators exsheath under the influence of the stain, it is evident that not all nonskin penetrators are quickly killed, as has been pointed out by Schwartz (16) and confirmed by the writer in experiments with larvae of *Obeliscoides cuniculi*.

With reference to the exsheathing process of the infective larvae of *Ancylostoma duodenale* in the presence of methyl green, Looss (11) states that "the experiment only succeeded beneath the cover glass, and even under these circumstances not unless a comparatively thin layer of fluid was allowed to remain between the cover glass and slide." Goodey (5) repeated this experiment with *Necator* larvae and obtained similar results. The writer was not able to confirm these observations in experiments with the infective larvae of *Ancylostoma caninum*. When a 1 per cent basic fuchsin solution was allowed to run in under a cover-slip preparation containing live larvae, the larvae were found to exsheath while a considerable quantity of fluid was still present between the slide and cover slip, when the larvae were immersed in a small vial containing a column of stain about 1.5 mm. high they exsheathed readily, despite the absence of pressure such as is afforded in a cover-slip preparation. The process of exsheathing in the latter case was observed under a binocular microscope.

#### EXPERIMENTAL INFECTION OF RABBITS WITH OBELISCOIDES CUNICULI

In order to determine the length of time required for *Obeliscoides cuniculi* to develop to fertile maturity in its rabbit host, a number of feeding experiments were carried out. In these experiments ensheathed infective larvae were fed to noninfected domestic rabbits by mouth, essentially to determine the location of the worms and the character of the lesions. The results, which likewise include some worm counts and other supplemental data, are given in Table 4.

TABLE 3—Summary of nine experiments on the resistance of infective *Obeliscoides cuniculi* larvae to air drying at room temperature <sup>a</sup>

Approximate number of larvae used	Length of exposure to drying	Condition of larvae after the addition of water
	Minutes	
15.....	5	All active
50.....	15	Do
50.....	30	15 dead, all the others active
50.....	60	8 active, all the others dead
30.....	120	2 active, all the others dead
30.....	180	4 moved spasmodically, all the others dead
30.....	240	1 moved spasmodically, all the others dead
30.....	300	All dead
30.....	360	Do

<sup>a</sup> Approximately 23° C

From Table 3 it is evident that the infective larvae of *Obeliscoides cuniculi* can withstand drying at room temperatures for several hours. Although some larvae succumbed after 30 minutes, others remained resistant for from 4 to 5 hours, however, the number of larvae that survived after 1 or more hours' exposure was relatively small. The survival period of *Obeliscoides cuniculi* infective larvae is longer than the survival periods of *Ancylostoma duodenale*, *Necator americanus*, *Monodontus phlebotomus*, and *M. trionocephalus*.

## REACTION TO LIGHT

In a glass jar containing a 15-day-old culture the larvae were found crawling up the walls of the jar facing the light of a north window, but no larvae were present on the opposite side of the jar. This indicates that the larvae reacted positively to diffuse daylight. One-half of the surface of a Petri dish containing mature larvae in water, more or less evenly distributed in the dish, was covered with black paper, and the other half, facing a northern window, was left uncovered. 24 hours later most of the larvae had collected in the lighted half of the dish. This observation is in harmony with that described above. One-half of a Petri dish containing larvae in water was covered with black paper, and the half that remained uncovered was illuminated with a bright electric light which was placed about 14 cm. from the dish. Four hours later most of the larvae were found in the shaded portions. This indicates that the larvae are repelled by strong artificial light.

In their reaction to light the infective larvae of *Obeliscoides cuniculi* behave like those of *Trichostrongylus* spp. (12) and *Haemonchus contortus* (19). Cameron (2) reported that larvae of *Monodontus* are positively thermotropic and are not repelled by direct sunlight, or even by an electric light.

## REACTION TO ANILINE STAIN

A drop of water containing about 25 larvae was put on a slide, and a cover slip was placed over it. A few drops of a 1 per cent aqueous solution of basic fuchsin were placed near one edge of the cover slip, so that the stain gradually filtered in and came in contact with the

Table 4 shows that after experimental feeding of *Obeliscoides* larvae the minimum time required for the worms to reach egg-laying maturity was 16 days (rabbits 6 and 7) and the maximum time was 20 days (rabbits 1 and 9). However, the appearance of eggs in the feces does not indicate that all the females present had reached the egg-laying stage. Rabbits 4, 8, and 9, which were killed and examined post-mortem 18, 31, and 21 days, respectively, after they had been fed infective larvae, contained a number of immature *Obeliscoides*, although the feces of these rabbits had contained eggs before necropsy. Of the larvae fed, the percentage that reached maturity, as shown by the number of worms recovered at necropsy, ranged from 31 per cent in rabbit 11 to 55 per cent in rabbit 10.

Rabbit 3, which had been infested with *Obeliscoides* for a period of more than five months, had at time of its death about 400 eggs per gram of feces. On post-mortem examination 214 males and 118 females were found in the stomach, of these females, 7 were gravid and the others had degenerated ovaries and uteri (fig 5), indicating that they had passed the stage of egg production and had become senile and sterile. The 7 gravid females yielded on an average 57 eggs per gram of feces for each egg-producing female. In contrast to this finding, rabbits 5 and 6, which harbored at necropsy only 39 and 92 adult females, respectively, a few days earlier had yielded 7,320 and 8,000 eggs per gram of feces, respectively. These observations show that the egg count is not necessarily a good index, in all cases, to the number of worms that an animal may harbor. The egg count gives no clue, for instance, to the presence of worms which are fully grown, but still agamic, nor to the presence of senile worms, neither does it account accurately for worm infestations in which there is a preponderance of males and for worm infestations in which, for one reason or another, there is a low egg production. By the use of the Stoll dilution technic it was found that rabbit 5 had 187 eggs per gram of feces for each of 39 female *Obeliscoides* harbored, 39 days after experimental infection and 3 days before the animal was killed. Rabbit 6, on the other hand, had only 87 eggs per gram of feces for each of 92 egg-producing females harbored, 50 days after experimental infection and 2 days before the animal was killed. This marked difference in egg production was

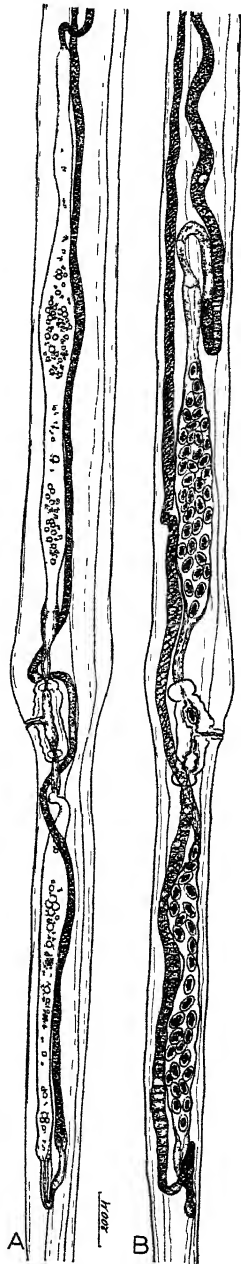


FIGURE 5.—Region of female reproductive organs of *Obeliscoides cuniculi*. A, Senescent female, B, gravid female

TABLE 4—Results of feeding infective *Obeliscoides cucuruli* larvae to domestic rabbits

Rabbit No	Date of feeding larvae	Larvae	Date of appearance of eggs in feces	Days elapsing from time of feeding	Date of necropsy	Days after infection	Number and maturity of worms recovered	Location of worms	Lesions noted in stomach
1.....	Sept 26, 1929	Number (c)	Oct 16, 1929	Number 20	Oct 26, 1929	Number 30	Numerous, <sup>b</sup> mature.	Several adult worms embedded in the stomach wall, the others free on the mucosa.	Petechial hemorrhages in mucosa
2.....	Sept 14, 1929	(*)	(*)	(*)	Nov 8, 1929	55	do.....	As above, several worms completely covered by mucus.	Hemorrhagic areas
3.....	Oct 26, 1929	(*)	(*)	(*)	Apr 5, 1930	101	214 males, 118 females, mature	Several worms embedded in the stomach wall, others free on the mucosa.	None
4.....	do.....	(*)	(*)	(*)	Nov 13, 1929	18	Numerous, <sup>b</sup> mature and immature	do.....	Congestion of mucosa and several large hemorrhagic areas
5.....	Nov 5, 1929	250	Nov 22, 1929	17	Dec 17, 1929	42	58 males, 38 females, mature	do.....	Congestion of mucosa and numerous petechial hemorrhages
6.....	do.....	500	Nov 21, 1929	16	Dec 27, 1929	52	72 males, 82 females, mature	do.....	Large masses of coagulated blood on stomach wall with numerous hemorrhagic areas
7.....	do.....	1,000	do.....	16	do.....	52	Numerous, <sup>b</sup> mature.	do.....	Wall of stomach coated with a fine layer of coagulated blood, several scattered hemorrhagic areas
8.....	do.....	2,000	Nov 23, 1929	18	Dec 6, 1929	31	946, mature and immature	do.....	Several petechial hemorrhages, with a few blood clots
9.....	do.....	2,700	Nov 25, 1929	20	Nov 26, 1929	21	Numerous, <sup>b</sup> mature and immature	do.....	Do
10.....	Dec 13, 1929	100	Dec 30, 1929	17	Jan 6, 1930	24	33 males, 22 females, mature	do.....	Do
11.....	do.....	200	do.....	17	Jan 11, 1930	29	41 males, 22 females, mature	Not recorded.	Not recorded
12.....	Dec 16, 1930	(*)	(*)	(*)	Dec 22, 1930	6	Numerous, <sup>b</sup> immature	Several worms embedded in stomach wall, others free on the mucosa.	Congestion of mucosa with numerous petechial hemorrhages

<sup>a</sup> Undetermined<sup>b</sup> Not counted

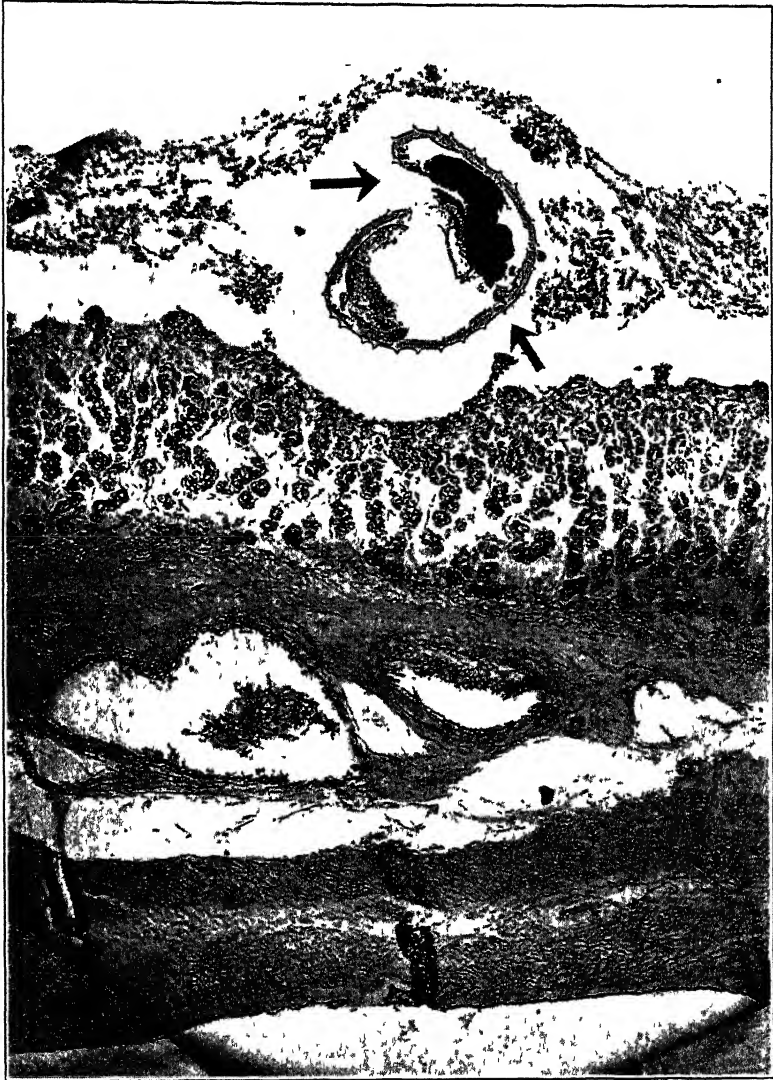


FIGURE 7—Cross section of the stomach wall of a rabbit showing section of *Obeliscoides cuniculi* (indicated by arrows) on the mucosa. Note erosion of the gastric glands. This rabbit was experimentally infected September 14, 1929, and examined post-mortem November 8, 1929.

probably due to the difference in the age of the worms; the worms in rabbit 6 were probably past their prime in egg production

The post-mortem examination of experimentally infected rabbits usually showed congestion of the gastric mucosa with numerous petechial hemorrhages. Erosion of the gastric glands and of the blood vessels was commonly observed in experimentally infected rabbits, large masses of coagulated blood also were found in the stomach



FIGURE 6—Stomach of rabbit infested with *Obeliscoides cuniculi*. This rabbit was experimentally infected September 26, 1929, and examined post-mortem October 26, 1929

contents of such rabbits. The worms were usually free on the mucous membrane or deep in the stomach wall. Histological examination of infected areas of the stomach wall revealed worms under the mucous membrane, some worms were noted in the submucosa. In microscopic section, the gastric glands were found to be eroded (Figs. 6, 7, and 8.)

susceptible to infestation with this nematode. The percentage of larvae that reached sexual maturity in guinea pigs was considerably lower than in most of the rabbits which were experimentally infected.

Lesions were observed in the stomachs of guinea pigs 3 and 7, in one of which a relatively large number of worms became mature. In guinea pigs 4B and 5, in which a relatively large number of immature worms were present, no stomach lesions were noted. In contrast to these observations, rabbit 12, in which the worms were also immature, had well-marked stomach lesions. Whether the rabbit is more susceptible to the injurious effects of *Obeliscoides* than the guinea pig remains to be determined.

TABLE 5—Results of feeding infective *Obeliscoides cuniculi* larvae to guinea pigs

Guinea pig No	Date of feeding larvae	Larvae fed	Date of necropsy	Days after infection	Number and description of worms recovered	Lesions noted in stomach
1.....	Dec 16, 1929	Number (°)	Jan 11, 1930	Number	1 gravid female.....	None
2.....	.....do.....	(°)	Feb 19, 1930	26	1 male.....	Do
3.....	Jan 20, 1930	200	Jan 22, 1930	65	58 third-stage larvae...	Congestion of mucosa, with numerous petechial hemorrhages
4A.....	.....do.....	200	Jan 25, 1930	5	Number undetermined, immature	None
4B.....	.....do.....	200	Jan 27, 1930	7	75 fourth-stage larvae...	Do
5.....	.....do.....	200	Feb 1, 1930	12	7 males and 16 immature females	Do
6.....	.....do.....	200	Feb 13, 1930	24	None.....	Do
7.....	Jan 27, 1930	200	.....do.....	17	17 males and 22 gravid females	Congestion of mucosa, with scattered petechial hemorrhages
8.....	Nov 25, 1930	(°)	Dec 22, 1930	27	None.....	None
9.....	Oct 17, 1930	(°)	Nov 7, 1930	21	1 male.....	Do
10.....	.....do.....	(°)	Dec 22, 1930	66	None.....	Do

° Several hundred

On several occasions the feces of infected guinea pigs containing *Obeliscoides* eggs were cultured by mixing the feces with animal charcoal and adding several drops of water to the mixture. The development of the eggs and larvae in such cases was normal.

#### STAGES OF LARVAL DEVELOPMENT IN THE GUINEA PIG

In connection with the study of the susceptibility of guinea pigs to *Obeliscoides*, parasites in various stages of development were recovered from these animals after experimental infection.

Guinea pig 3, which was killed two days after experimental infection, yielded third-stage larvae; these worms showed the commencement of the third molt, as indicated in Figure 9. The principal measurements of six of these larvae were as follows: Total length,  $856\mu$  to  $1,013\mu$ , maximum width,  $26\mu$  to  $30\mu$ ; length of esophagus,  $186\mu$  to  $213\mu$ ; distance of nerve ring from the anterior end,  $114\mu$  to  $121\mu$ ; distance of excretory pore from the anterior end,  $133\mu$  to  $159\mu$ ; length of tail,  $57\mu$  to  $76\mu$ . The most significant morphological feature of these larvae, as shown in Figure 9, was the position of the genital

EXPERIMENTAL INFECTION OF GUINEA PIGS WITH *OBELISCOIDES CUNICULI*

In order to determine the adaptability of *Obeliscoides* to a host other than the rabbit, a similar series of experiments was conducted which involved experimental infection of guinea pigs with infective larvae of this parasite. The results are given in Table 5.

Table 5 shows that *Obeliscoides* larvae can be successfully transmitted to guinea pigs. As far as can be determined from a survey of the literature, *Obeliscoides* has not been transmitted to guinea pigs heretofore. Of the larvae fed to guinea pigs, only a few succeeded in



FIGURE 8—Cross section of stomach wall of rabbit showing sections of *Obeliscoides cuniculi* (indicated by arrows) in the deeper part of the submucosa

reaching sexual maturity. In guinea pigs 3 and 4B, which were killed 2 and 7 days, respectively, after infection, more than one-fourth of the larvae that had been fed were recovered at necropsy, the worms thus recovered were still immature. From the findings on guinea pigs it may be assumed that the worms are eliminated from these animals to a large extent before reaching maturity. Guinea pig 7, which was fed about 200 larvae, contained 39 mature worms at necropsy, indicating that this particular animal was rather highly

320 $\mu$  Females—total length, 4.8 to 5 mm, maximum width, 98 $\mu$  to 120 $\mu$ , length of esophagus, 483 $\mu$  to 530 $\mu$ ; distance of nerve ring from the anterior end, 197 $\mu$  to 250 $\mu$ , distance of excretory pore from the anterior extremity, 319 $\mu$  to 349 $\mu$ , distance of vulva from the anus, 748 $\mu$  to 936 $\mu$ , combined lengths of ovejectors 190 $\mu$  to 230 $\mu$ ; length of tail 114 $\mu$  to 121 $\mu$

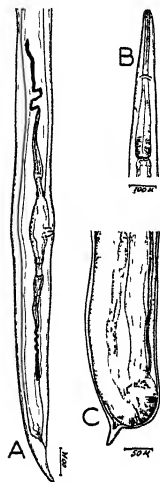


FIGURE 11—Fourth-stage larvae of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig seven days after experimental infection. A, Posterior end of female, B, anterior end of female, C, posterior end of male

Guinea pig 5, killed 12 days after experimental infection, yielded fifth-stage worms or adults (Fig 12). The males were unsheathed, a fact which indicates that the fourth molt had been completed and that the worms were in their final stage, the bursa and its rays resembled those of the adult forms, and the spicules were chitinized. The females, however, still retained the fourth or final sheath. The genital organs had developed considerably beyond the previous stage, the sphincters of the ovejectors and uteri were well developed and the ovaries were longer than in the previous stage and somewhat coiled. The ovaries were beginning to show developing ova, the posterior lobe of the ovary was looped, as shown in Figure 12.

The principal measurements of three males and three females were as follows. Males—length, 5.2 to 6 mm, maximum width, 125 $\mu$  to 156 $\mu$ , length of esophagus, 490 $\mu$  to 639 $\mu$ , distance of nerve ring from the anterior end, 234 $\mu$  to 270 $\mu$ , distance of excretory pore from the anterior end, 390 $\mu$  to 421 $\mu$ , width of spread-out bursa, 140 $\mu$  to 171 $\mu$ , length of spicules, 440 $\mu$  to 452 $\mu$ , corresponding with those of the adult forms. Females—length, 5.5 to 6.2 mm, maximum width, 202 $\mu$  to 218 $\mu$ , length of esophagus, 624 $\mu$  to 655 $\mu$ , distance of nerve ring from the anterior end, 265 $\mu$  to 280 $\mu$ , distance of excretory pore from the anterior end, 390 $\mu$  to 436 $\mu$ ; distance of vulva from anus, 1,014 $\mu$  to 1,138 $\mu$ , combined lengths of ovejectors, 234 $\mu$  to 265 $\mu$ , length of tail, 109 $\mu$  to 147 $\mu$ .

Guinea pig 7, which was killed 17 days after experimental infection, yielded adult worms of both sexes. Most of the females had segmenting eggs in the uterus, whereas the uteri of others contained no eggs.

The period required for *Obeliscoides* to reach egg-laying maturity in guinea pigs is about the same as that in rabbits. Development of *Obeliscoides* in both of these hosts, therefore, proceeds at about the same rate.

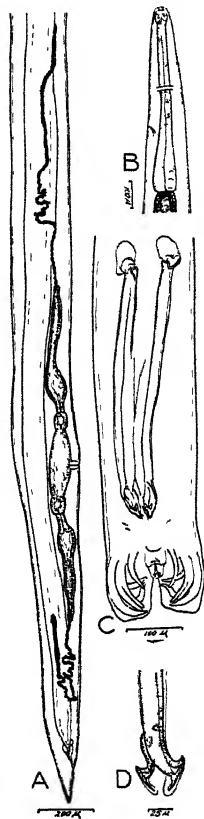


FIGURE 12—Fifth-stage adults of *Obeliscoides cuniculi* recovered from the stomach of a guinea pig 12 days after experimental infection. A, Posterior end of female, B, anterior end of female, C, posterior end of male, D, lateral view of tips of the right spicule

primordium, which had migrated posteriorly and was located approximately  $170\mu$  from the anus, in the particular specimen which was drawn. The position of the genital primordium in the various larvae

recovered from this guinea pig varied, being from  $80\mu$  to  $363\mu$  from the anus.

Guinea pig 4A, which was killed 5 days after experimental infection, yielded fourth-stage larvae in which sex differentiation had become definitely established. The female reproductive apparatus showed considerable development. The vulva, ovejectors, uteri, and ovaries were definitely recognizable, as shown in Figure 10. At this stage of development the males may be distinguished by the inflated posterior end, which eventually forms the bursa.

The principal measurements of one female were as follows: Length, 2.26 mm, maximum width,  $68\mu$ ; length of esophagus,  $357\mu$ , distance of nerve ring from the anterior extremity,  $121\mu$ , distance of vulva from the tip of the tail,  $440\mu$ , combined lengths of female reproductive apparatus, about  $350\mu$ , length of tail,  $87\mu$ .

Guinea pig 4B was killed seven days after experimental infection. This animal yielded fourth-stage females and males. The males were approaching the final molt, as evidenced by the sheath which is well separated from the body (Fig. 11). The bursa and its rays were still incompletely developed. The spicules were incompletely developed and only partly chitinized. The ejaculatory duct and the testis, the two being continuous, were fairly well developed. At this stage the ejaculatory duct is a slender tube opening into the cloaca and extending anteriorly for a short distance until it joins the testis. The latter is ventral to the intestinal tract. The females were less advanced in development than the males, they did not possess a sheath, a fact which indicates that they were not as yet approaching the final molt. The female genital apparatus at this stage is

FIGURE 9—Third stage larva of *Obeliscoides cumuli* recovered from the stomach of a guinea pig two days after experimental infection.



clearly differentiated into a pair of ovejectors, sphincters, uteri, and ovaries (Fig. 11). The principal measurements of these worms were as follows: Males—length, 4.3 to 4.8 mm, maximum width,  $114\mu$  to  $130\mu$ ; length of esophagus,  $483\mu$  to  $530\mu$ ; distance of nerve ring from the anterior end,  $182\mu$  to  $220\mu$ , distance of excretory pore from the anterior extremity,  $273\mu$  to  $326\mu$ , length of spicule, approximately

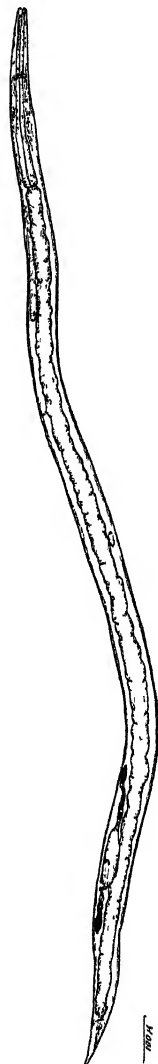


FIGURE 10—Fourth-stage larva of *Obeliscoides cumuli* recovered from the stomach of a guinea pig five days after experimental infection.

- (5) GOODEY  
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- (6) ———  
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## SUMMARY

In common with those of other strongyles, the free-living larvae of *Obeliscoides cuniculi* undergo two molts in fairly rapid succession. In charcoal cultures and at room temperatures the infective stage is reached in about six days.

The infective larvae failed to produce skin lesions and subsequent infestations when placed on the intact skin of live rabbits. These larvae were incapable of burrowing into the skin of young rats under experimental conditions.

Infective larvae were found to withstand a temperature of 2° to -4° C for 30 days, but the vitality of most of them was destroyed after being kept at a temperature of -18° C for 3 days.

The infective larvae did not appear to be very resistant to desiccation, as all of them were found dead after a 5-hour exposure to air drying at room temperature.

The infective larvae responded positively to diffuse daylight but were repelled by strong artificial light.

In the presence of a 1 per cent solution of basic fuchsin, the infective larvae did not exsheath and remained active in the stain for a period of 18 hours.

Most of the infective larvae reached sexual maturity in the stomach of rabbits in 16 to 20 days.

Post-mortem examination of experimentally infected rabbits, within about a month after the larvae were fed by mouth, usually revealed areas of inflammation in the gastric mucosa and the presence of petechial hemorrhages and blood clots on the stomach wall. The worms were found free on the mucosa or embedded in the stomach wall.

The larvae undergo two molts in the stomach of the rabbit before attaining sexual maturity, the worms become sexually differentiated after the third molt, but the bursa is not fully developed until the fourth or final sheath has been cast off.

In several instances infective larvae were found to reach sexual maturity in the stomach of guinea pigs; in such cases the eggs developed normally and the larvae reached the infective stage after undergoing the usual two molts. The time required for *Obeliscoides* larvae to reach the egg-laying stage in the guinea pigs was about the same as in the rabbits.

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# SEASONAL SUBSOIL TEMPERATURE VARIATIONS<sup>1</sup>

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## INTRODUCTION

Actual diurnal fluctuations of temperatures follow the geometric progression law, and as found by Bouyoucos (2, p. 131)<sup>2</sup> "the diurnal-nocturnal amplitude of oscillation of temperature decreased in geometric progression as the depth increased in arithmetic progression, in all the different types of soil" Keen (10, p. 308), in discussing the theory of heat flow in a conducting material, emphasized the importance of the heat conductivity of the soil, which he defined "as numerically equal to the quantity of heat flowing per unit of time through unit area of a unit cube of material when unit temperature difference is maintained between two opposite faces" He also considered the specific heat, the apparent density, and the diffusivity of the soil, and assuming the application to the soil surface of a simple harmonic variation, showed how the amplitude of such a temperature wave diminished exponentially as the depth of observation increased

Smith (17, 18) has reported that a distinct rise and fall in soil temperatures in a 24-hour period occurs to a depth of 12 inches, and the night temperatures for the 6-inch and 12-inch depths average higher than the day temperatures The diurnal range in soil temperatures is influenced largely by the character of the sky, rainfall or soil moisture, and the direction and intensity of the wind (17) The highest soil temperatures in the surface soil as reported by Schucht (15) in Germany, are about 113° F, in the Russian steppes 140°, and in western Arizona 160° Smith (16) reported that the highest temperature at Davis, Calif, in an area kept free from vegetation, and obtained at a depth of one-half inch, was 143°.

The soil temperatures that have usually been reported have been those occurring on the soil surface or at depths less than 3 feet McClatchie (12) conducted soil-temperature investigations in the Salt River Valley of Arizona by the use of three self-registering thermometers situated underground at depths of 5, 10, and 15 feet. His results during the two years of investigation show that the annual range of temperature decreases with depth, for at 5 feet it varied from 20° to 25° F., at 10 feet, from 15° to 20°, and at 15 feet, from 10° to 15° He estimated that in the area under investigation, at a depth of about 50 feet, the soil temperature would remain constant throughout the year Rambaut (14) determined the monthly temperatures in a gravelly soil under grass at Oxford, England, and found that the annual soil temperature range at a depth of 10 feet was 9.5°.

Callendar and McLeod (4) at Montreal reported soil temperatures from an area covered with a layer of turf, where the soil consisted of loose, light-brown sand to a depth of 8½ feet, below which to a depth

<sup>1</sup> Received for publication Sept. 9, 1931 issued April, 1932

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 427



## LOCAL CONDITIONS AND PROCEDURE

The area in which the soil temperatures were determined is located at Davis, Calif., on a recent alluvial fan which has a slope in the immediate vicinity of from 5 to 10 feet per mile. The texture of the soil in the various horizons is loam from the surface to a depth of 3 feet, fine sandy loam from 3 to 9 feet, coarse and fine sand from 9 to 11½ feet, and silt loam to 12 feet.

The rains in this district occur mainly between September and May, and as the area has been kept free from vegetation since 1923, and the water table has remained at about 20 feet, the soil is moist to field capacity during most of the winter season. During the dry season (May to September), as the area is not irrigated, the moisture content of the surface foot of soil is reduced by surface evaporation, but at greater depths the changes are slight. The moisture changes which usually occur in this area during the year have previously been described (17).

In November, 1928, electrical resistance thermometers were installed at depths of 4, 5, and 6 feet, and in September, 1929, at 8, 10, and 12 feet. The thermometer bulb consists of an insulated nickel winding inserted in a strong brass tube, serving as a case. The lead-covered leads are soldered with a water-proof joint to the stem of the thermometer and connected to a cable, which extends to a small building near the plots, where a resistance thermometer indicator of the balance type is located. In burying the thermometers, a hole of small diameter was dug, the soil of the various horizons being carefully laid aside. The thermometers were then inserted and the hole filled, the soil layers being put back in proper order and lightly tamped in order to attain the same degree of compactness as existed originally. The soil temperatures at these depths change very slowly and weekly readings were therefore sufficient.

## INTERPRETATION OF TEMPERATURE DATA

Subsoil temperatures at the same depth vary from year to year. During certain parts of the year the subsoil temperatures may remain fairly constant for several weeks, and for this reason 4-week averages were determined for various depths ranging from 4 to 12 feet, inclusive. These averages are shown in Figure 1. In Figure 1, particular attention is called to the data obtained in 1930, when during November the temperatures did not vary appreciably in the 4 to 12 foot depths. It appears that May and November are the pivotal months of the year. The time of occurrence of the minimum and maximum average soil temperatures as related to depth are clearly illustrated.

Taking the weekly readings, it was found that the temperature ranges in 1930 at the various depths, as shown in the lower right-hand corner of Figure 1, were 27° F. at 4 feet, 22° at 5, 18° at 6, 14° at 8, 12° at 10, and 9° at 12 feet. With increasing depth the amplitude of temperature changes per foot of depth were of lesser magnitude in the deeper subsoil than in the upper subsoil.

Air temperatures were obtained in a standard United States Weather Bureau shelter at Davis, Calif., near the area where the subsoil temperatures were determined. The lowest air temperature in 1929, which was 23° F., was recorded on February 11. The mean

of about 30 feet there was a bed of stiff blue clay. Water was always found in the sand for a certain distance above the clay. The annual ranges in soil temperature were 34.3° F at a depth of 20 inches, 26.0° at 40 inches, 19.5° at 66 inches, and 11.0° at 108 inches. The area in which these observations were taken was covered with snow from approximately January 1 to April 1.

Fitton and Brooks (8) have summarized the data which have been reported on soil temperatures in the United States up to 1931 in various soils under different conditions of cover and moisture at different elevations and exposures, and have made available in their discussion a very complete bibliography of literature on this subject.

### ROOT GROWTH AND SOIL TEMPERATURE

As the roots of certain plants, under favorable soil conditions, extend to depths greater than 3 feet, and during the summer months such roots in the deeper areas are in a soil climate cooler and in the winter warmer than their aboveground parts, it was deemed advisable to obtain soil temperatures to a depth of 12 feet.

The seasonal subsoil temperature changes even at depths of 12 feet are important, as a rise of temperature is brought about by the physical process of absorption, conduction, diffusion, and convection (7). A lowering of the temperature is brought about by a reciprocal process involving diffusion, conduction, vaporization, and radiation. The characteristics of the solid, liquid, and gaseous phases of the soil mass affect the rate of this movement.

The most favorable soil temperature for most crops, if other conditions are favorable, is usually considered as being between 65° and 70° F. (19). Roots in the deeper soils grow vigorously at much lower temperatures, varying of course with the species (20). Cannon (5) found that the most rapid rate of root growth in seedlings of *Prosopis velutina* occurred at a soil temperature of about 93.2°, at which point roots with an initial length of 16 mm grew 51 mm in 12 hours. He found that not only was the rate of growth of shoots correlated with the temperature of the soil, but also with the length of the roots.

Under a diminished oxygen supply the effect of soil temperature seems to be greatly modified (6). As the oxygen supply in the soil is diminished, the rate of growth diminishes in a soil with a high temperature. In other words, crops in order to attain a fair rate of growth in time of high soil temperatures, must be in a well-aerated soil; otherwise, the rate of growth is considerably reduced. One of the most effective agents in soil ventilation is the changing daily or seasonal soil temperatures. The volume of a given mass of air is increased or diminished by  $\frac{1}{491}$  of its original volume for each degree Fahrenheit change in temperature, the pressure remaining constant. Under such conditions a fall in temperature of 1° F. throughout a volume of 491 cubic feet of air would cause the entrance into the soil of 1 cubic foot of air. An increase in temperature of an equal amount would result in the movement outward of the same quantity of air (11). The effect of temperature upon soil aeration is not due, however, merely to the expansion and contraction of gases, but also to their differential absorption (1) by the soil at different temperatures. Alfalfa, red clover, field peas, and soybeans have been found to give a maximum nodule production at a soil temperature of about 75.2° (9).

4 feet for some months—May, October, November, and December—were in part interpolated. In January the upper subsoil has the lowest temperature and the soil is progressively warmer in the lower depths. In the month following, the upper subsoil becomes warmer while the deeper subsoil becomes cooler. The lowest average monthly temperatures for the lower subsoil (12 feet) is in April, while at a depth of 1 foot the average April temperature is  $60^{\circ}\text{F}$  as compared to  $47^{\circ}$  in January. It will be noticed further that the average April temperatures are nearly the same ( $60^{\circ}$ ) for all depths from 6 inches to 12 feet.

The upper subsoil continues to become warmer until July, and then becomes cooler in August, while the deeper subsoil is warmer in August than in July. In fact, the highest average monthly temperatures at 10 feet occur in October, and at 12 feet the average temperatures for October and November are practically the same. The upper subsoil cools rapidly from September to January. Keen (10) in discussing Rambaut's results has pointed out that the periodic nature of the temperature wave closely approaches a 6-monthly symmetry.

The subsoil temperatures, particularly at depths of 4 feet or more, show more accurately the total amount of heat absorbed by the soil than the temperatures at depths less than 4 feet, as the latter are more greatly affected by fluctuations in air temperatures. The heat conductivity into the soil is also illustrated from 10-year averages obtained in bluegrass sod in Illinois (13) where the highest average monthly temperature ( $73.5^{\circ}\text{F}$ ) at a depth of 9 inches was reached in July, while at a depth of 36 inches the highest average monthly temperature ( $67.5^{\circ}$ ) was reached in August.

In Table 1 the monthly mean air temperatures and the rainfall for 1930 and the departures from the normal are shown. The normal monthly mean temperatures and rainfall are based on records extending for 23 to 59 years, respectively. The monthly mean temperatures reported (3) were determined from maximum and minimum thermometers exposed in a standard United States Weather Bureau shelter  $4\frac{1}{2}$  feet above the surface of the soil, a short distance from the soil-temperature plots.

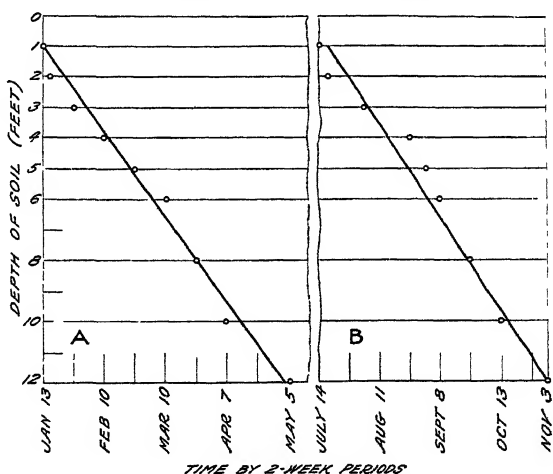


FIGURE 2.—Occurrence of minimum (A) and maximum (B) soil temperatures at depths varying from 1 to 12 feet, Davis, Calif., 1930

monthly temperature for January of that year was  $40.4^{\circ}$ , and for February  $46.9^{\circ}$ . The highest air temperature in 1929 was  $111^{\circ}$ , on June 25. The lowest air temperature during the first part of 1930 was  $24^{\circ}$ , on January 13, and the highest during the year was  $107^{\circ}$ , on July 14. The time of occurrence of the minimum and maximum soil temperatures in 1930, at depths ranging from 1 foot to 12 feet, is practically a straightline function of the depth (Fig 2). The minimum at the 1-foot depth occurred on the same day as the minimum air temperature, while at a depth of 12 feet the minimum did not occur until May 5, or 16 weeks later. In like manner, the maximum temperature at the 1-foot depth in 1930 occurred shortly after the maximum air temperature, while at a depth of 12 feet the maximum was

not reached until November 3, or 15 weeks later.

The time of the occurrence of the minimum and maximum soil temperatures as compared to the minimum and maximum air temperatures, or the lag, is not the same each year. During some years this lag at the 3-foot depth may be 80 hours (17), while in 1930 it ranged from 2 to 3 weeks. This is due to the varying moisture content of the soil and is also affected by the character of the air temperature preceding and following the time of occurrence of the minimum and maximum temperature. In other words, during the week preceding or following the occurrence of the maximum air temperature, the air temperatures may have been as much as 10 degrees lower or perhaps within 2 degrees of the maximum.

The changing soil temperatures throughout the year can also be illustrated by determining the mean monthly temperatures (fig 3), and in this way the ebb and flow of heat can be better understood. As previously stated, the soil temperatures at depths of 4 to 12 feet, inclusive, were obtained by weekly readings.

During most of 1930 continuous temperature records were also obtained for various depths less than 4 feet by means of electrical resistance thermometers and an automatic temperature recorder.

From data obtained during previous years and by the use of mean monthly air temperatures, the average monthly soil temperatures were determined for those months in 1930 when the automatic temperature recorder was not operated. The data, therefore, for depths less than

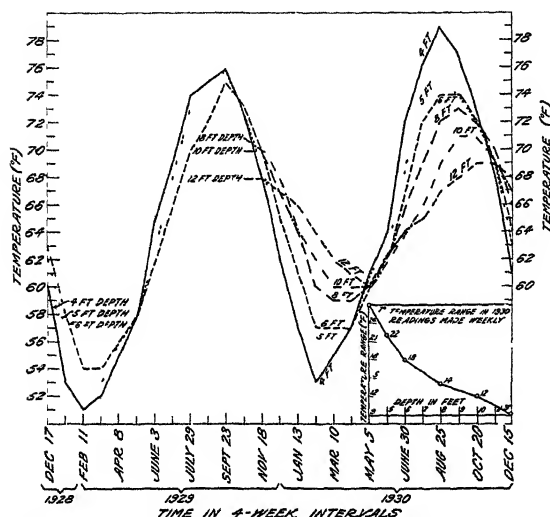


FIGURE 1—Subsoil temperatures by 4-week averages at depths varying from 4 to 12 feet, Davis, Calif., December 17, 1928, to December 15, 1930

## SUMMARY

Soil temperatures were determined by means of electrical resistance thermometers at Davis, Calif., in an unirrigated area that was kept free from vegetation during the experiment and for 6 years before the experiment. The water table stood at a depth of 20 feet. During the wet season, September to May, the soil was generally moist to field capacity. During the dry season the greatest loss of moisture occurred from the surface foot of soil.

During 1930, May and November appeared to be pivotal months with relatively uniform soil temperatures at all depths from 4 to 12 feet. The annual temperature ranges for the various depths were 27° F at 4 feet, 22° at 5 feet, 18° at 6 feet, 14° at 8 feet, 12° at 10 feet, and 9° at 12 feet. In the lower subsoil the temperature ranges per foot increase in depth were not of such great magnitude as in the upper subsoil.

The time of occurrence of the minimum and maximum soil temperatures at depths ranging from 1 foot to 12 feet is practically a straight-line function of the depth. At the 1-foot depth they occurred on the same day as the minimum and maximum air temperatures, while at a depth of 12 feet the minimum soil temperature did not occur until 16 weeks later, and the maximum not until 15 weeks later.

The average monthly soil temperatures for depths ranging from 6 inches to 12 feet show that during the early part of the year the upper subsoil has the lowest temperature and the subsoil is progressively warmer with the depth. Although the upper subsoil becomes progressively warmer in February, March, and April the lower subsoil becomes progressively colder, attaining in April its lowest average monthly temperature for the year. In April the average temperature (60° F) is nearly the same for all depths from 1 foot to 12. The upper subsoil continues to become warmer until July, and then becomes cooler in August, while the deeper subsoil is warmer in August than in July. At a depth of 12 feet, the highest average temperatures occur in October and November. A 6-monthly symmetry is shown by the temperature waves.

The annual average soil temperatures for depths ranging from 6 inches to 12 feet were found to vary from 65° to 67° F, while the annual mean air temperature, based on records obtained from minimum and maximum thermometers, was 58.8°.

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TABLE 1—Monthly mean air temperatures and rainfall, with departures from the normal, at Davis, Calif., 1930

Month	Monthly mean air temperature	Departure from normal	Monthly rainfall	Departure from normal
	° F	° F	Inches	Inches
January.....	43.6	-1.0	3.80	-0.07
February.....	52.0	+3.4	1.66	-1.15
March.....	54.0	+1.1	3.48	+1.07
April.....	57.2	0	.92	-.17
May.....	59.0	-4.1	.18	-.45
June.....	70.6	+6	0	-.14
July.....	72.7	-1.9	0	-.01
August.....	73.0	-.2	0	-.01
September.....	65.6	-3.6	.23	-.10
October.....	59.3	-2.1	.69	-.03
November.....	53.6	+1.8	.92	-.69
December.....	44.6	0	.20	-3.20
Annual mean.....	58.8	-5	12.08	-4.95

The monthly mean air temperatures in 1930 for February, March, June, and November were higher than the normal, and during the remaining months of the year they were either equal to, or fell below, normal. In February, 1930, the mean monthly air temperature was 3.4° F. higher and in May it was 4.1° lower than the normal. It was during these two months that the greatest departures in the air temperatures from the normal occurred.

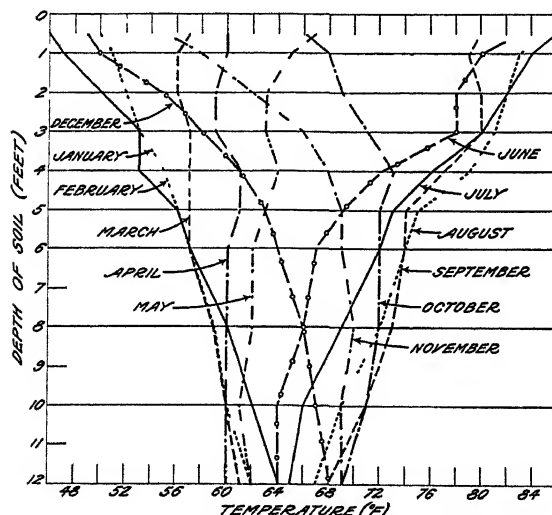


FIGURE 3—Monthly averages of soil temperatures in degrees Fahrenheit at depths varying from 1 to 12 feet, Davis, Calif., during 1930

The rainfall from the normal occurred in December of 1930, when the precipitation was 3.20 inches below normal.

The annual average soil temperatures for depths varying from 6 inches to 12 feet for the year 1930 were found to range from 65 to 67° F. At 6 inches, 1 foot, and 12 feet the average was 65°, at 3 feet and 10 feet it was 67°, and for the other depths it was 66°. The average annual mean air temperature, based on records obtained from minimum and maximum thermometers, is 59.3°, and during 1930 it was 58.8°. The annual average soil temperatures for 1930, at all depths from 6 inches to 12 feet, were therefore higher than the mean air temperature for 1930 determined from minimum and maximum thermometers.

# A METHOD FOR THE DETERMINATION OF COMPARATIVE HARDINESS IN SEEDLING ALFALFAS BY CONTROLLED HARDENING AND ARTIFICIAL FREEZING <sup>1</sup>

By GEORGE L PELTIER, *Plant Pathologist, Nebraska Agricultural Experiment Station*, and H M TYSDAL, *Associate Agronomist, Division of Forage Crops and Diseases, United States Department of Agriculture*

## INTRODUCTION

In a recent publication the writers (5) <sup>2</sup> pointed out that 2-year-old, field-grown alfalfa plants were not found to be satisfactory for use in comparative controlled-hardiness tests. The variability of both the environmental factors and the plants within any one sort was so great that comparisons between different alfalfas varying in hardiness within small limits could not be made. Striking differences, however, were obtained between hardy, midhardy, and nonhardy alfalfas. In order to decrease this variability materially and shorten the time element, seedling alfalfa plants, grown entirely under controlled conditions in the greenhouse, were studied to determine whether or not they can be employed in comparative hardiness tests.

The primary object of this investigation was to develop adequate methods under controlled conditions that would give (1) consistent hardiness values comparable to field results, (2) a suitable procedure for the selection of hardy plant types, and (3) standard conditions that would serve as a basis for fundamental studies concerning the nature of hardiness in alfalfa and other crop plants.

## EQUIPMENT

The studies herein reported were carried out with the controlled hardening and freezing equipment recently described by Peltier (3). The hardening chamber was maintained at a uniform temperature slightly above 0° C. The temperatures in the freezer room were varied, depending on the temperature and length of exposure desired. The temperature in the freezer and the temperature of the materials under test were determined with copper-constantan thermocouples by means of a potentiometer and galvanometer. In later experiments a 16-point Leeds and Northrup resistance thermometer recorder was substituted for the thermocouples.

## METHODS OF PLANTING AND GROWING THE SEEDLINGS

### GENERAL METHODS AND PROCEDURE

It was found in preliminary trials that hard seeds sometimes caused confusion in the survival counts, especially when young seedlings were tested, because these seeds germinated after the artificial freezing. To avoid this complication small quantities of all alfalfa seeds, as needed, were treated for 10 minutes with concentrated sulphuric acid.

<sup>1</sup> Received for publication Sept. 21, 1931, issued April, 1932. Paper No. 109 of the Journal Series of the Nebraska Agricultural Experiment Station. This paper, the second of a series on hardiness in alfalfa, is based on cooperative investigations between the Department of Plant Pathology, Nebraska Agricultural Experiment Station, and the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

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counts of the plants can be made two weeks after freezing. All final counts in the cold-resistance tests were made two weeks after the plants were exposed to low controlled temperatures.

INFLUENCE OF THE TYPE OF CONTAINER ON THE VARIABILITY OF THE PERCENTAGE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

The following four types of containers were employed in these studies: (1) Cypress flats (15 by 18 by 6 inches), (2) metal water-tight flats of the same dimensions; (3) porous clay pots (4 inches in diameter), and (4) square metal water-tight cans (4 by 4 by 6 inches). The plants were grown in these several types of containers to determine the type most suitable for comparative cold-resistance tests, particularly from the standpoint of least variability in percentage survival of the seedlings after artificial freezing. The plants used in these experiments were uniformly 1 month old and had been hardened for 15 days before freezing.

The standard deviation of a single determination, the coefficient of variability, and Weinberg's coefficient of variability of the percentage of survival of the seedlings after freezing are given in Table 1 for the different types of containers. The standard deviation of a single determination of all the tests which were extremely low or extremely high in percentage survival of the seedlings is less than that of the tests with intermediate percentages of survival. The average standard deviation for all containers with a survival from 76 to 100 per cent is 9.1, and from 0 to 24 per cent is 10.6, whereas the standard deviation for all containers with a survival from 25 to 75 per cent is 24.8. This is to be expected, since a very low or very high percentage of survival of the seedlings tends to eliminate actual variations because the freezing temperatures may be so high that none of the seedlings are killed or they may be so low that all the seedlings are killed. In either case there would be no variability.

TABLE 1.—*The influence of the type of container on the variability of the percentage survival alfalfa seedlings after exposure to low controlled temperatures*

PLANTS IN CYPRESS FLATS

Kind of alfalfa	Average survival	Replications <sup>a</sup>	Standard deviation of a single determination	Coefficient <sup>b</sup> of variability	Weinberg's <sup>c</sup> coefficient of variability
	<i>Per cent</i>	<i>Number</i>			
Turkestan.....	77.0	24	20	26	4.2
	47.0	8	24	51	5.6
	41.0	8	29	71	6.2
	32.0	8	19	59	5.0
	29.0	8	20	69	5.2
Nebraska common.....	28.0	8	20	71	4.9
	24.0	8	21	88	5.6
	5.0	8	7	140	3.6
Arizona common.....	2.0	8	4	200	3.0
	6	8	1	167	1.2

<sup>a</sup> 15 to 30 plants per replication in the large containers and 10 to 20 plants per replication in the small containers, month-old plants hardened uniformly for 15 days at 2° to 3° C. previous to freezing, length of exposure and temperature varied with each test, depending on the type of container.

$$^b C V = \frac{\sigma}{M} \times 100$$

<sup>c</sup>  $W = \frac{\sigma \sqrt{Mn - Mo}}{\sqrt{(Ma - Mo)(Mn - Ma)}}$  where M=mean, Mn=the highest value, Mo=the lowest value, Ma=mean of all variants

and immediately thereafter thoroughly washed with tap water and spread to dry.

Enough soil was prepared at the beginning of each year for the entire season. This consisted of finely shredded sod soil to which some sand was added. This mixture was uniform in texture and high in fertility, so that it was possible to adjust soil moisture readily and grow healthy and vigorous plants.

Several methods of seeding were followed, depending on the type of container and the nature of the experiment. As a rule, all sowings were heavier than necessary, and the plants were later thinned. In the small containers the seed was sown broadcast. If there were fewer than eight plants in a container it was discarded. The small containers usually had from 10 to 20 plants, the average being about 15. Apparently the difference in the number of plants per container within these limits was not important, as no decrease in variability occurred when the plants in the pots were thinned to a uniform number.

Two methods were used in sowing the seeds in the large containers: (1) Sowing broadcast a different alfalfa in each of the four quarters of the flats, and (2) sowing the seed in rows lengthwise of the flats and thinning ordinarily to 20 to 30 plants per row. Precautions were taken not to sow the seeds too near the outer edges of the flats.

The writers have been unable to find any publications dealing with comparative hardiness tests of young alfalfa seedlings under controlled conditions. For the most part previous investigators (5) have employed plants grown either in the greenhouse or field at least six months or longer, and in no instance were the plants subjected to controlled hardening. In this study all seedlings were grown under optimum conditions in the greenhouse in several types of containers usually for about one month, but occasionally for two. The plants were then placed in a hardening chamber for periods of 2 to 39 days and subsequently exposed to low controlled temperatures, so that in general the age of the plants at the time of the artificial freezing tests did not exceed 8 weeks.

In all tests the plants were counted just before they were transferred to the hardening chamber and again after they had recovered from freezing. Thus, a percentage of survival based on the total number of plants was obtained. A system of determining relative survival or injury by observation, as employed by Hill and Salmon (1), was not adopted because actual counts were considered more accurate. No notes on injury to the tops were taken, for generally the tops were completely killed back to the crown, from which the new growth usually started.

A preliminary series of tests was carried out to determine when survival counts should be made after the plants were artificially frozen. The new growth was observed in some instances in less than one week after freezing. It was found that at the end of two weeks all plants that had survived the artificial freezing recovered and produced sufficient new growth to be readily counted. Subsequent counts made at periods up to four weeks after freezing did not materially change the final number, although usually a slightly lower survival was found at the end of four weeks because a few plants died after initial recovery. The average of 36 readings showed a 40 per cent survival two weeks after freezing and a 38 per cent survival four weeks after freezing. The results of these tests show that reliable

flats, it appears to make little difference so far as variability in the percentage survival is concerned, which type of container is employed. A serious objection, however, to the use of metal flats is indicated below.

TABLE 2—*The influence of the various types of containers on the variability in survival of 1-month-old alfalfa seedlings after exposure to low controlled temperatures<sup>a</sup>*

Kind of alfalfa	Type of container	Average Weinberg's coefficient of variability
Turkestan.....	(Cypress flats.....)	5.6
	Metal flats (constant moisture).....	3.9
	Metal cans (constant moisture).....	5.2
	Pots.....	6.7
Grimm.....	Metal cans (constant moisture).....	6.1
	Pots.....	5.6
	Pots (constant moisture).....	6.0
Nehraske common.....	Cypress flats.....	5.2

<sup>a</sup> Only the data from the experiments reported in Table 1 which showed a survival of 25 to 75 per cent were averaged.

#### INFLUENCE OF THE POSITION OF THE ROW IN THE FLATS ON THE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

In order to determine whether the position of the row in the flats had any influence on the survival of seedlings after freezing, the following experiment was undertaken. Seed of Turkestan alfalfa was sown in 6 rows lengthwise in 4 cypress and 4 metal flats. The seedlings were allowed to grow under optimum conditions for one month in the greenhouse before they were transferred to the hardening chamber. Here they remained for two weeks and were then exposed to freezing temperatures. Two weeks later a count of the surviving plants was made. In the cypress flats the percentages of survival were as follows. In the 2 inner rows 87.6, in the 2 intermediate rows 79.9, and in the 2 outer rows 63.9 per cent; in the metal flats the corresponding percentages were 55.8, 35.4, and 19.4. The difference in the percentage survival between the two inner and the two outer rows in the metal flats was much greater (36.4 per cent) than in similar rows in the cypress flats (23.7 per cent).

In order to determine whether there was more variability in survival between plants in the rows of one flat than between rows in different flats, calculations were made from the data obtained in the foregoing study. It was found that the standard deviation calculated by the deviation-from-the-mean method on the basis of six rows within each of 4 cypress flats was 13.9, whereas between rows in the same relative position in different flats it was 20.2. Similar results for the metal flats gave standard deviations of 21.1 and 16.1, respectively. These results show that variability in survival within the same cypress flat is less than that between rows in the same relative position of different flats frozen at the same time. The reverse is true of the variability of survival in the metal flats. The variability within a cypress flat is less than in any of the other combinations. Since it is desirable in comparative cold-resistance tests that comparisons between unknown alfalfas and the control be as accurate as possible, the cypress flats were extensively employed and alfalfas of

TABLE 1—*The influence of the type of container on the variability of the percentage survival of alfalfa seedlings after exposure to low controlled temperatures*—Continued

PLANTS IN METAL FLATS (CONSTANT MOISTURE)					
Kind of alfalfa	Average survival	Replications <sup>a</sup>	Standard deviation of a single determination	Coefficient <sup>b</sup> of variability	Weinberg's <sup>c</sup> coefficient of variability
	Per cent	Number			
Turkestan.....	57	24	16.1	44	3.9
PLANTS IN METAL CANS (CONSTANT MOISTURE)					
	95.0	6	3	3	2.1
	87.0	6	4	4	2.3
	86.0	7	8	9	4.4
Turkestan.....	75.0	7	8	11	3.1
	71.0	7	26	36	6.6
	67.0	7	23	34	5.6
	74	7	29	39	6.6
Grimm.....	59	25	27	46	5.5
	17	10	20	118	5.6
PLANTS IN POROUS CLAY POTS					
	94	8	7	7	3.3
	84	8	11	13	4.4
	70	8	31	44	6.9
Turkestan.....	54	8	18	33	5.1
	48	8	30	62	6.0
	46	8	23	50	5.3
	45	8	47	104	9.4
	25	8	31	124	7.2
	85	8	13	15	4.2
Grimm.....	74	8	39	54	8.9
	58	8	11	19	2.2
PLANTS IN POROUS CLAY POTS (CONSTANT MOISTURE)					
(19 mm.....)	94	7	7	7	3.4
	74	8	25	31	6.0

It is evident from the data in Table 1 that the coefficient of variability does not offer a dependable measure of the variability of the surviving population, because in general the higher the percentage survival the lower the coefficient of variability. Weinberg's coefficient of variability as used by Winter (7) is not subject to this criticism, since it is not dependent upon the magnitude of the percentage survival of the seedlings after artificial freezing. In comparing the influence of the type of container on the variability of the percentage survival, Weinberg's coefficients were averaged in only those instances in which survival was between 25 and 75 per cent. The data shown in Table 2 indicate that the variations of the variability in percentage survival in the different containers were not large. With Turkestan seedlings there was least variability in the metal flats and cans, while with Grimm there was least in the pots. The metal containers always had a weighed amount of soil which was brought to a constant moisture content before freezing. An attempt was also made to have the moisture content of the soil in the cypress flats and pots as nearly uniform as possible before freezing. With the possible exception of the metal

Since the freezing in this experiment was too severe to bring out differences in the younger plants, a second experiment was undertaken to ascertain the behavior of the plants at these stages of growth when exposed to less severe freezing. A series of plantings of two alfalfas was made at intervals in pots. At the end of 26 days after the first seeding the entire lot was placed in the hardening chamber and kept there for 15 days. At the end of that time the plants in the pots were exposed for three and one-half hours to a temperature of  $-14^{\circ}\text{C}$ . Two weeks later survival counts were made. (Table 4.) Apparently the seedlings were somewhat more resistant to cold in the cotyledon stage than when the third leaf had developed, although there were no appreciable varietal differences in cold resistance at these stages. As the trifoliate leaves appeared, the seedlings were not only more cold resistant but the varietal response to cold became more marked. It might be mentioned that the stage of development as shown in Table 4 also applies in general to plants of the same approximate age in Table 3. In comparative cold-resistance tests, therefore, the seedlings were grown for approximately one month under optimum conditions, at which time at least three true leaves had developed.

TABLE 4—*Influence of age and stage of development on the cold resistance of alfalfa seedlings*<sup>a</sup>

Kind of alfalfa	Age of seedlings previous to hardening	Stage of plant development	Average survival <sup>b</sup>
	Days		Per cent
Turkestan.....	26	2 to 3 trifoliate leaves.....	69 0
Arizona common.....	26	do.....	43 0
Turkestan.....	19	1 trifoliate leaf.....	43 0
Arizona common.....	19	do.....	18 0
Turkestan.....	10	Cotyledons and third leaf.....	5 1
Arizona common.....	10	do.....	4 b
Turkestan.....	5	Cotyledons emerging.....	13 5
Arizona common.....	5	do.....	13 3

<sup>a</sup> All plants in pots hardened uniformly for 15 days at  $2^{\circ}$  to  $4^{\circ}\text{C}$ , and exposed for  $3\frac{1}{2}$  hours to a temperature of  $-14^{\circ}\text{C}$ .

<sup>b</sup> Average of 9 replications, each replication consisting of 15 to 30 plants of each alfalfa at each stage of development.

## METHODS OF HARDENING SEEDLINGS

The importance of the hardening process in crop plants has been established through the studies of many investigators. In the present work the writers were interested particularly in determining the optimum condition for hardening and the exposure which would produce maximum hardening of alfalfa seedlings. For these studies the hardening chamber (3) was maintained at a uniform temperature of about  $2^{\circ}$  to  $4^{\circ}\text{C}$ ., no attempt being made to vary it.

## INFLUENCE OF THE LENGTH OF EXPOSURE IN THE HARDENING CHAMBER ON THE SURVIVAL OF SEEDLINGS AFTER ARTIFICIAL FREEZING

Since the time required for maximum hardening in seedling alfalfas under controlled conditions has apparently never been reported, this constituted one of the essential steps in the development of a comparative test for cold resistance. A number of experiments were undertaken to determine this point.

unknown hardness were sown in alternate rows in the same flat with an alfalfa of known hardness. The percentage survival of the three rows of unknown and the three rows of known alfalfas were averaged, thus eliminating any systematic error with reference to the original position of the seedlings in the flats.

#### INFLUENCE OF AGE ON THE RESISTANCE OF SEEDLINGS TO COLD

To determine at what age young alfalfa seedlings would exhibit comparable differences in resistance to cold, the following experiment was undertaken with Turkestan, Grimm, and Arizona common. Triplicate seedlings of each alfalfa were made in rows in cypress flats each succeeding week for a period of two months under optimum conditions for growth. At the end of this period all flats were transferred to the hardening chamber maintained at a temperature of 2° to 4° C and kept there for 15 days. Since a large number of flats were involved, all could not be exposed to low temperatures at the same time, so that it was necessary to freeze on three successive days. The average exposure was six hours at -16°. After freezing, the plants were removed to the greenhouse, and two weeks later survival counts were made. The percentage of survival of the different alfalfas at various ages is shown in Table 3. Apparently, under the conditions of this experiment, seedlings up to the age of 18 days are very susceptible to cold, all alfalfas being almost completely killed out. Comparative differences in cold resistance are well marked whether the seedlings are 25 or 60 days old. The percentage of survival at 32 days is fairly high, although not so high as that of older seedlings.

TABLE 3—*Influence of age on the cold resistance of alfalfa seedlings*\*

Kind of alfalfa	Age of seedlings previous to hardening	Average survival <sup>b</sup>	
	Days	Per cent	Per cent
Turkestan.....	60	79	51
Grimm.....		51	
Arizona common.....		21	
Turkestan.....	53	82	61
Grimm.....		67	
Arizona common.....		34	
Turkestan.....	47	51	23
Grimm.....		16	
Arizona common.....		1	
Turkestan.....	39	33	20
Grimm.....		26	
Arizona common.....		1	
Turkestan.....	32	43	25
Grimm.....		23	
Arizona common.....		8	
Turkestan.....	25	23	14
Grimm.....		14	
Arizona common.....		6	
Turkestan.....	18	2	1
Grimm.....		0	
Arizona common.....		0	
Turkestan.....	11	0	0
Grimm.....		0	
Arizona common.....		0	
Turkestan.....	4	0	0
Grimm.....		0	
Arizona common.....		0	

\* All plants hardened uniformly for 15 days at 2° to 4° C, and exposed for 6 hours to a temperature of -16° C.

<sup>b</sup> Average of three replications, each replication consisting of 15 to 30 plants of each alfalfa at each stage of development.

Only one alfalfa, Tukestan, was employed in the second experiment of this series. Seed was sown in 85 pots at the same time, and eight pots were removed at various intervals to the hardening room. After hardening, the plants were exposed on the same day for 6½ hours to a temperature of  $-14.6^{\circ}\text{C}$ . This temperature was somewhat lower than that employed in experiment 1, and consequently the percentages of survival were lower. The results are shown in experiment 2, Table 5. All the plants that were transferred directly from the greenhouse to the freezer room without hardening succumbed. Even the plants that were held in the hardening chamber for eight days failed to recover. However, four additional days in the hardening chamber resulted in a survival of about 34 per cent. Sixty-five per cent of the plants that had had a hardening period of 16 days recovered. Extending the period up to 23 days apparently did not materially increase the percentage of survival.

A third experiment with Grimm seedlings was similar to the others, except that the length of exposure in the hardening chamber was extended to 35 days. Seed for the plants hardened 35 days were sown a month before the others. The plants were exposed on the same day to a temperature of  $-14.6^{\circ}\text{C}$ , but for only 5½ hours. In general, the results are similar to those reported in the preceding experiment, namely, the percentage survival after artificial freezing is directly correlated with the length of the hardening period, within certain limits. It will be noted that only 4 per cent survival resulted when the hardening period was extended to 35 days, and it is apparent that a prolonged period of hardening so weakens the young seedlings that they are easily killed by artificial freezing. In these three experiments the plants, with one exception, were all of the same age at the time of freezing, but they varied in the stage of development, since they were transferred to the hardening chamber at different intervals.

Up to this point the study of the influence of hardening on cold survival under controlled conditions has involved seedlings grown in pots. Although the seedlings were the same age, they had not reached the same stage of development when exposed to artificial freezing, because the different groups of seedlings remained in the hardening chamber for different lengths of time. A fourth experiment was therefore made in which cypress flats were used and the dates of planting were so arranged that the plants would have reached the same stage of development at the time they were transferred to the hardening chamber. Briefly, three flats were sown to each of five alfalfas on three different dates. While the plants were all of the same age when they were placed in the hardening chamber, 1 lot was hardened for 39 days, 1 for 18 days, and 1 for 8 days. The plants in all the flats were then exposed for 8 hours to a temperature of  $-17^{\circ}\text{C}$ . The length of exposure was greater and the temperature was lower in this experiment than in the pot experiments, since larger amounts of soil were contained in the flats than in the pots. However, the results are similar to those already given, as may be noted in experiment 4, Table 5. Here again, as in experiment 3, an extended hardening period apparently so weakened the plants that they could not withstand artificial freezing. It is interesting to note that the plants in experiments 1, 2, and 3, Table 5, that were in the hardening chamber longest, were able to overcome

In the first experiment 50 pots of each of 5 alfalfas—Turkestan, Grimm, Nebraska common, Utah common, and Arizona common were planted in October and allowed to grow in the greenhouse for 35 days. Four pots of each of the 5 alfalfas, containing from 10 to 20 plants, were then transferred to the hardening chamber, first at 3-day intervals, then at 2-day intervals, and finally at 1-day intervals. All plants of the five alfalfas were then exposed to a temperature of  $-13.6^{\circ}\text{C}$  for four to seven hours. The percentage of plants surviving at the end of two weeks is shown in Table 5, experiment 1. The percentage of survival was highest for the plants that had previously had a 14-day hardening period, and lowest for the plants that had been in the hardening room only 4 to 6 days. In general, the results show that, particularly with the hardier sorts, the longer the plants are held in the hardening room the higher the percentage of survival after artificial freezing and apparently the wider the differences between the alfalfas representing hardy, midhardy, and nonhardy types.

TABLE 5—*Influence of the length of exposure in the hardening chamber on the survival of alfalfa seedlings to artificial freezing*

PLANTS IN POTS (EXPERIMENT 1) <sup>a</sup>					
Days in hardening chamber (number)	Percentage survival of—				
	Turkestan	Grimm	Nebraska common	Utah common	Arizona common
14	93	94	75	53	40
11	69	69	69	72	73
9	78	67	57	48	29
7	53	40	22	25	5
6	45	48	31	26	23
5	27	15	14	25	8
4	35	28	7	0	0
PLANTS IN POTS (EXPERIMENT 2) <sup>b</sup>					
23	66	-----	-----	-----	-----
19	62	-----	-----	-----	-----
16	65	-----	-----	-----	-----
12	34	-----	-----	-----	-----
8	0	-----	-----	-----	-----
0	0	-----	-----	-----	-----
PLANTS IN POTS (EXPERIMENT 3) <sup>b</sup>					
35	-----	1	-----	-----	-----
14	-----	98	-----	-----	-----
11	-----	80	-----	-----	-----
6	-----	43	-----	-----	-----
2	-----	11	-----	-----	-----
PLANTS IN CYPRESS FLATS (EXPERIMENT 4) <sup>c</sup>					
39	15	18	5	15	1
18	82	60	58	43	16
8	61	55	43	20	12

<sup>a</sup> Each figure represents 4 pots containing 10 to 20 plants of each alfalfa, plants exposed for from 4 to 7 hours at  $-13.6^{\circ}\text{C}$ .

<sup>b</sup> Each figure represents 8 pots containing 10 to 20 plants, the Turkestan plants were exposed for  $6\frac{1}{2}$  hours and the Grimm plants for  $5\frac{1}{2}$  hours to a temperature of  $-14.6^{\circ}\text{C}$ .

<sup>c</sup> Each figure represents three flats containing 5 alfalfas, of about 30 plants each, plants exposed for 8 hours at  $-17^{\circ}\text{C}$ .

TABLE 6—*Relation of the hardening and freezing temperatures, and the soil temperature during freezing, to the percentage survival of alfalfa seedlings*<sup>a</sup>

Soil temperature before freezing	Period of exposure	Temperature maintained	Resulting soil temperatures at depths of—			Survival of alfalfa plants
			13 cm	38 cm	76 cm	
° C	Hours	° C	° C	° C	° C	Per cent
19.5	2	-18.3	5.3	10.0	12.0	47
4.0	2	-18.3	-1.1	6	9	75
18.0	4	-10.0	1.3	6.0	8.7	50
6.0	4	-10.0	4	1.1	1.7	100

<sup>a</sup> Based on a study of 4 flats containing 15 to 30 1-month-old seedlings of each of 4 alfalfas

The air temperatures in the freezer room exert a decided influence on the rate of freezing and the ultimate survival of the seedlings. An air temperature of  $-18.3^{\circ}$  C. for two hours resulted in a 47 per cent survival, whereas a 4-hour exposure at  $-10^{\circ}$  was required to produce approximately the same percentage survival in those flats with a high soil temperature previous to freezing. It will later be shown that the final soil temperature during freezing likewise has an important bearing on the subsequent survival of the seedlings. In other words, all these factors are important and each must be considered in determining the proper procedure in comparative freezing tests.

#### INFLUENCE OF THE PERCENTAGE OF SOIL MOISTURE ON THE RATE OF FREEZING IN THE SOIL AND PERCENTAGE SURVIVAL OF SEEDLINGS

In the preceding section it was shown that soil temperatures at the time the flats were placed in the freezer influenced the rate of freezing the soil. In these experiments moisture content of the soil was uniformly high. In order to determine the influence of the percentage of soil moisture on the rate of freezing, four alfalfas (Grimm, Nebraska common, New Mexico common, and California common) were sown in each of four flats and allowed to grow for one month under optimum conditions. They were then transferred to the hardening chamber for a period of two weeks. Just before freezing, the soil was allowed to dry out, so that it contained approximately 12 per cent moisture in all the flats. The soil in three flats was then brought to 17, 27, and 33 per cent moisture, and the soil in the fourth allowed to remain at 12 per cent. The flats were then exposed for various periods to an average temperature of  $-18.9^{\circ}$  C.

By means of thermocouples placed in the soil at a depth of 13 centimeters the rate of depression of the soil temperature was obtained. After freezing, the flats were transferred to the greenhouse, and two weeks later survival counts were made. The results of this experiment are presented graphically in Figure 1. In the soil with a moisture content of 12 and 17 per cent the temperatures dropped to  $0^{\circ}$  C. within a few minutes, and within four hours had reached temperatures of  $-5^{\circ}$  and  $-4^{\circ}$ , respectively. On the other hand, in the soil with a moisture content of 27 per cent, the temperature at the end of 4 hours was still above  $0^{\circ}$  and it did not reach  $-4^{\circ}$  until after a 10-hour exposure, a difference of 6 hours. Finally, in the flat having a soil moisture of 33 per cent, the temperature did not reach  $0^{\circ}$

the handicap of being somewhat less advanced in development, which, as Tables 3 and 4 show, would naturally make them less resistant to cold. This fact emphasizes the large degree of hardening which occurs during the longer periods.

From these experiments it may be concluded that 1-month-old alfalfa seedlings not only attain maximum hardening in about 15 days under controlled temperatures of 2° to 4° C., but that these same conditions bring out the greatest differences between the hardiness of the various alfalfas. A hardening period of a few days or one of prolonged duration is not satisfactory, because comparative differences are not so pronounced. Therefore, in comparative hardiness tests 1-month-old seedling alfalfas were held in the hardening chamber for two weeks prior to artificial freezing.

#### METHODS OF FREEZING THE SEEDLINGS

For the artificial freezing of alfalfa seedlings a freezer room (3) was used in which uniform temperatures at any point between 0° and -30° C. could be maintained for indefinite periods. Before a comparative cold-resistance method could be developed, it was necessary to determine the length of exposure to temperatures which would insure a uniform percentage of survival of the same alfalfa in repeated tests and bring out the maximum differences in survival between alfalfas of different degrees of hardiness.

#### INFLUENCE OF THE HARDENING AND FREEZING TEMPERATURES AND THE SOIL TEMPERATURE DURING FREEZING ON PERCENTAGE SURVIVAL OF SEEDLINGS

As the alfalfa seedlings were exposed to low temperatures either in small or large containers, a study of the influence of the various factors involved in determining the correct length of exposure and temperature for a uniform survival of the same alfalfa was necessary. Some of the factors which may be involved are: (1) The soil temperature prior to freezing; (2) the air temperature and length of exposure in the freezer, (3) the rate of freezing of the soil; and (4) the relative importance of the air and soil temperatures on the survival of the seedlings. Flats containing 6 inches of soil and 1-month-old alfalfa seedlings were held at different temperatures prior to freezing. The soil temperature was measured by means of thermocouples at three depths after an exposure for different periods of time and also at two freezer-room temperatures. The results of the various temperature measurements, together with the percentage survival obtained under these conditions, are listed in Table 6. The results show that the soil temperatures previous to freezing have a direct bearing on the degree of lowering of the soil temperatures in the freezer and the subsequent survival of the seedlings. For example, the flats with the high soil temperatures before freezing had a much greater drop in soil temperature than those with a low soil temperature before freezing. This is reflected in the lower percentages of survival. A contributing influence, however, is the degree of hardening which occurred in those plants exposed to the lower temperatures before freezing. This hardening enabled a greater number of plants to survive, even though the final soil temperature was lower than that in the other flats.

consistent results than a long exposure to a higher temperature. Numerous trials have indicated that records of the soil temperature depressions aid greatly in determining the proper freezing exposure. Better results have been obtained when the containers were exposed for relatively short intervals at a low temperature than when they were left until the soil had reached the air temperature of the freezer room. This necessitates longer exposures at higher temperatures. The former procedure is more nearly comparable to conditions in the field, where minimum soil temperatures rarely approach minimum air temperatures. Therefore, in most of the comparative hardiness trials temperatures between  $-10^{\circ}$  and  $-20^{\circ}$  C were employed, with relatively short exposures. No definite freezing exposure can be given, as this will vary with the degree of hardening of the seedlings as well as with soil type and other factors.

The length of exposure to a given low temperature, however, is to some extent reflected in the percentage of survival, as shown in Table 7. The percentages of survival were obtained by averaging the number of 1-month-old plants in five pots of each of eight alfalfas hardened off for a 2-week period, and exposed for various intervals to a temperature of  $-14.5^{\circ}$  C. An hour and twenty minutes of exposure to this temperature resulted in a survival of 72 per cent, while an exposure of  $3\frac{1}{2}$  hours reduced the survival to 20 per cent. For each increase in time of exposure between these two points there was a definite decrease in the number of seedlings that survived.

TABLE 7—*Influence of the length of exposure to a temperature of  $-14.5^{\circ}$  C.<sup>a</sup> on the percentage survival of alfalfa seedlings*

Length of exposure		Average survival of alfalfa plants
Hours	Minutes	Per cent
1	20	72
1	50	61
3	00	33
3	30	20

<sup>a</sup> Based on a study of 5 pots containing approximately fifteen 1-month-old plants, of each of 8 alfalfas, 1, e, 600 plants. The plants had been hardened for 15 days.

#### METHOD OF HANDLING THE SEEDLINGS AFTER FREEZING

In practically all the experiments reported, the plants were removed directly to the greenhouse from the freezer room. From 24 to 36 hours elapsed before the soil in the flats came to the greenhouse temperature. Investigators are still in disagreement as to whether the rate of thawing is a factor in the differential responses of alfalfas after artificial freezing. Janssen (2) states that slow thawing increases the percentage of survival in wheat, Weimer (6) reports that slow thawing does not increase the percentage survival in alfalfa. The writers (5) have presented data which show that slow thawing of 2-year-old alfalfa plants increased the percentage of survival.

In an experiment one set of seedlings in pots was returned directly to the hardening chamber from the freezer room and held for a period of eight days before removal to the warm greenhouse, while a second set was taken immediately from the freezer room to the greenhouse.

until after a 5-hour exposure, and from this point the temperature gradually dropped to  $-2.2^{\circ}$  at the end of over 15 hours.

Thus the drier the soil, the faster was the temperature depressed. The higher the moisture content of the soil, the more gradual was the temperature depression. The lowest temperatures were also recorded in the drier soil. In spite of the shorter exposure, the percentages of survival were lower in the dry soil and progressively higher as the moisture content of the soil increased.

These results show that the influence of the moisture content of the soil is pronounced, not only on the rate and final temperature depression in the soil, but also on the percentage survival of the plants. Thus, in comparative hardness tests with seedling alfalfas

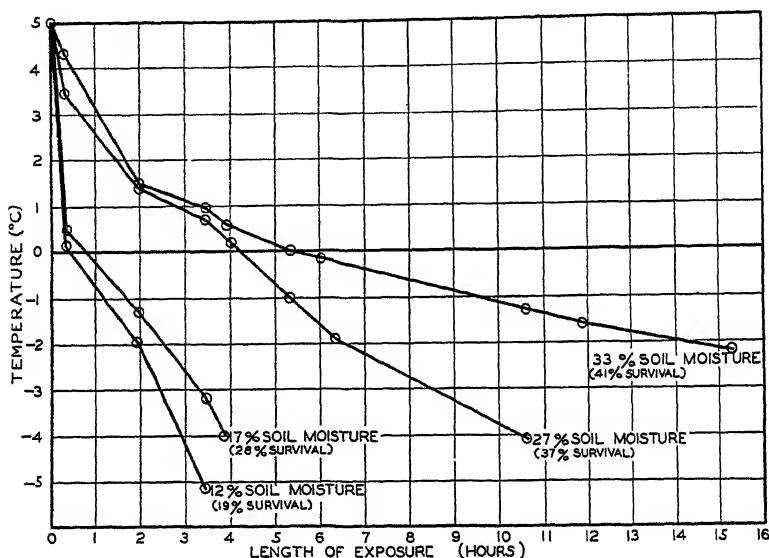


FIGURE 1.—Rate of temperature depression in the soil, as influenced by the soil-moisture content, and subsequent percentages of survival of alfalfa seedlings exposed in flats to a temperature of  $-18.9^{\circ}\text{C}$  for different periods of time. The 1-month-old seedlings were hardened for two weeks at the same soil moisture, which was adjusted just prior to freezing.

it is imperative that not only the temperature of the soil but also the moisture content be made as uniform as possible before the seedlings are frozen. A comparatively high soil moisture is to be preferred because a more uniform and gradual temperature depression occurs and the exposure can be extended over longer periods. In all comparative hardness tests the moisture of the soil in which the seedlings are growing is made relatively high and uniform in all flats exposed at one time in the freezer room.

#### INFLUENCE OF LENGTH OF EXPOSURE TO LOW TEMPERATURES ON PERCENTAGE SURVIVAL OF SEEDLINGS

The technic of freezing seedlings has not been so perfected that it is possible to state that an exposure to a stated low temperature for a definite period will result in a certain percentage of survival. Neither have enough records been accumulated to show whether a short exposure to a low temperature will give more uniform and

TABLE 9—Comparative cold resistance of alfalfas<sup>a</sup>

Kind of alfalfa	Survival of plants—			
	In flats		In pots	
	Per cent	P E <sup>b</sup>	Per cent	P E <sup>b</sup>
Turkestan (F C I No 15754).....	72	±7 3	70	±1 5
Grumm (F C I No 15713).....	58	±5 9	68	±5 3
Nebraksa common.....	51	±5 2	53	±5 6
Utah (F C I No 15815).....	32	±3 3	50	±5 9
Arizona (F C I No 15837).....	14	±1 4	40	±5 7

<sup>a</sup> Based on average results from 6 flats, each flat containing 15 to 30 plants of each alfalfa, and from 20 pots containing 10 to 20 plants. Plants were grown for 1 month, hardened for 15 days, flats were exposed for 6 hours to a temperature of  $-16^{\circ}\text{C}$  and pots for  $5\frac{1}{2}$  hours to  $-13^{\circ}\text{C}$ .

<sup>b</sup> P E of the mean, calculated by deviation-from-the-mean method

In order to test the validity of the method further, a series of eight alfalfas of known hardiness were sown both in pots and in rows in cypress flats. The seedlings were allowed to grow for one month in the greenhouse. Those in the flats were then hardened for 13 days and those in the pots for 11 days. The flats were then removed to the freezer room and exposed for  $2\frac{3}{4}$  hours to a temperature of  $-20.4^{\circ}\text{C}$ . To insure success in obtaining the correct freezing exposure, the pots were divided into four lots of five pots each for each alfalfa. The first lot was exposed for  $1\frac{1}{2}$  hours, the second for  $1\frac{1}{2}$ , the third for 3, and the fourth for  $3\frac{1}{2}$  hours to a temperature of  $-14.4^{\circ}$ .

The percentage of seedlings surviving after these exposures in the freezer room are given in Table 10, together with the probable errors, calculated by the deviation-from-the-mean method. The higher probable errors in the tests with flats can be accounted for by the fact there were only four replications, whereas with the pots there were 20. This experiment, therefore, gives some idea of the number of replications necessary for comparative testing for cold resistance of alfalfas.

TABLE 10—Comparative cold resistance of alfalfas

Kind of alfalfa	F C I accession No	Survival of plants—			
		In flats <sup>a</sup>		In pots <sup>b</sup>	
		Per cent	P E <sup>c</sup>	Per cent	P E <sup>c</sup>
Ladak.....	14135	47	±8 9	78	±1 9
Turkestan.....	15754	46	±8 7	65	±1 6
South Dakota common.....	14210	22	±4 2	56	±2 6
Kansas common.....	15749	8	±1 5	45	±3 5
New Mexico common.....	14470	3	± 6	42	±2 5
Provence.....	15744	2	± 4	29	±2 0
Arizona.....	15837	1	± 2	33	±2 3
Peruvian.....	15830	0	-----	24	±1 5

<sup>a</sup> Based on 4 replications of 15 to 30 1-month-old plants for each alfalfa, hardened for 13 days, and exposed for  $2\frac{3}{4}$  hours to a temperature of  $-20.4^{\circ}\text{C}$ .

<sup>b</sup> Based on 20 replications of 10 to 20 1-month-old plants for each alfalfa, hardened for 11 days, and exposed for from  $1\frac{1}{2}$  to  $3\frac{1}{2}$  hours to a temperature of  $-14.4^{\circ}\text{C}$ .

<sup>c</sup> P E of the mean, calculated by deviation-from-the-mean method

The agreement between the rank of the alfalfas tested in the flats and pots is very close, and corresponds very nearly with the relative hardiness found with these same alfalfas in field experiments. During the past season (1930-31), individual hardiness tests have been made on 100 different sorts of alfalfa and, in general, as judged by

At the end of two weeks the average percentage survival of those held in the hardening chamber for eight days and thawed slowly was  $32 \pm 1.9$  per cent as compared to  $18 \pm 1.5$  per cent for those which were thawed rapidly. Good comparable results were obtained by following a uniform practice in removing the plants directly from the freezer to the greenhouse. It yet remains to be determined whether there is a differential varietal response to slow thawing which would serve to bring out greater differences.

#### APPLICATION OF THE METHOD IN COMPARATIVE COLD RESISTANCE TESTS WITH ALFALFA

In developing methods for testing comparative cold resistance, a number of alfalfas, representing different degrees of hardness, were employed in order to compare the results with those that would be expected under field conditions with the same sorts of alfalfa. As a rule the kinds used had been tested in general agronomic practice for a period of years, so their hardness was known.

In the first tests three alfalfas of different degrees of hardness were employed. Each alfalfa was grown in 24 replications in rows in flats, so that there were approximately 480 plants of each alfalfa. The seedlings were grown for 1 month in the greenhouse, transferred to the hardening chamber for 13 days, and then exposed to a temperature of  $-19.6^{\circ}\text{C}$  for  $8\frac{1}{2}$  hours. The percentages of survival are listed in Table 8. They are significantly different and in the same order as shown in field tests.

TABLE 8—*Comparative cold resistance of alfalfas* <sup>a</sup>

Kind of alfalfa	Survival of plants	
	Per cent	P E <sup>b</sup>
Turkestan (F C I No 15754).....	40	$\pm 3.4$
Nebraska common.....	27	$\pm 2.8$
Arizona (F C I No 15837).....	3	$\pm .7$

<sup>a</sup> Based on a study of 24 replications of 15 to 30 plants per row. Plants were grown for 1 month in flats, hardened for 13 days, and exposed for  $8\frac{1}{2}$  hours to a temperature of  $-19.6^{\circ}\text{C}$ .

<sup>b</sup> P. E. of the mean, calculated by deviation-from-the-mean method.

In a second experiment five alfalfas were employed, and seed was sown in pots and in rows in flats. Each alfalfa was replicated six times with 15 to 30 plants in each row in the flats, and twenty times with each 10 to 20 plants in each pot. The plants in the two types of containers were grown in the greenhouse for 1 month, transferred to the hardening chamber for 15 days, and finally the flats were exposed for 6 hours to a temperature of  $-16.0^{\circ}\text{C}$  and the pots for  $5\frac{1}{2}$  hours to a temperature of  $-13.8^{\circ}$ . Two weeks after freezing, survival counts were made. (Table 9.) In general they give a relative percentage of survival comparable to that obtained in field tests. While the percentages of survival in the two types of containers are not always identical, because of different lengths of exposure to low temperatures, the results are in agreement in differentiating the degree of hardness between alfalfas. Some may question the placing of Turkestan above Grimm in the hardness scale. However, these results have been consistent in the writer's tests. The writers (4) have shown that certain lots of Turkestan are more hardy under the conditions of these tests than Grimm, whereas some are less hardy.

# INHERITANCE IN BARLEY<sup>1</sup>

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## INTRODUCTION

Although an abundance of genetic work has been done on animals, only a few plant species have been studied in detail. Barley, a crop of economic importance, has many easily determinable characters and a small number of chromosomes. For this reason it offers good material for inheritance studies. With the common occurrence of chlorophyll-defective seedlings the chance of identifying different character pairs has increased, and a large number of factor pairs has been added to those already known. The studies presented in this paper are a continuation of previous studies made for the purpose of establishing linkage groups in barley.

## REVIEW OF LITERATURE

A fairly extensive review of the literature on linkage relations in barley is now available (5, 6, 3, 1, 2)<sup>3</sup>. The character pairs discussed in this paper which have been studied by previous workers are shown in Table 1.

TABLE 1—Character pairs discussed in this paper which have been studied by previous workers

Character pair	Investigator	Number of factors involved	Symbol used
Rough v smooth awn.....	Hayes and Garber (5).....	Single-factor difference..	(Rr)
	Griffie (3).....	2-factor difference F <sub>1</sub> ratio, 12 3 1	(RrSs)
Style branching.....	Sigfusson (12).....	2-factor difference F <sub>2</sub> ratio, 9 3 3 1	(RrSs)
	Robertson and Deming (11)	3 pairs of cumulative factors	Gg, G'g', g''g''
Long v short haired rachilla.....	Hor (7).....	Single-factor difference..	(Ll)
	Robertson (10).....		(Ss)
	Sigfusson (12).....		(Ll)
	Buckley (1).....		(Ll)
Black v white glume color.....	Griffie (3).....	do.....	(Bb)
	Hayes and Garber (6).....		(Bb)
	Robertson (10).....		(Bb)
	Sigfusson (12).....		(Bb)
Blue v white aleurone.....	S6 and Imai (13).....	Single-factor difference (vena)	{ (Bl bl)
	Hayes and Garber (5).....		
	Buckley (1).....		
Hoods v awns.....	Hor (7).....	Single-factor difference..	(Kk)
	Hayes and Garber (5).....		(Kk)
	Robertson (10).....		(Kk)
	Buckley (1).....		(Kk)
Green v chlorina.....	Nilsson-Ehle (9).....	do.....	(Ff)
	Hallquist (4).....		(Ff)
Green v albino.....	Nilsson-Ehle (9).....	do.....	(Aa <sub>1</sub> a <sub>2</sub> )
	Hallquist (4).....		(Aa <sub>2</sub> )

<sup>1</sup> Received for publication Aug 8, 1931, issued April, 1932. The part of this paper dealing with the character differences in a Coast X Lion cross was submitted to the Department of Agronomy, Utah State Agricultural College, by Dwight Koonce in partial fulfillment of the requirements for the degree of master of science, March, 1931.

<sup>2</sup> The writers gratefully acknowledge the cooperation of Director C P Gillette and Prof. Alvin Kezer, of this station, in providing facilities for this investigation. The writers are also indebted to Dr. H K Hayes, professor of plant genetics, University of Minnesota, for helpful criticisms of the manuscript.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 466.

those whose hardness was known, the results have been uniform and consistent in giving an expression of their relative hardness

With a sufficiently large number of replications, the results obtained indicate the possibility of a wide application of this method. In addition to its adaptability for testing new strains, selections, and introductions, it would appear to be valuable for selecting harder types within a mass population

#### SUMMARY

The steps involved in the development of a method for the determination of comparative hardness in seedling alfalfas under controlled conditions have been presented. This method as developed up to the present time consists essentially of the following general procedure

Alfalfas are seeded in small pots or preferably in cypress flats in alternate rows with a control alfalfa of known hardness, and allowed to grow under optimum conditions in the greenhouse for one month. They are then transferred to the hardening chamber, held at a temperature of 2° to 4° C for two weeks. Before the seedlings are frozen, the soil is brought to a high and uniform moisture content. The flats with the seedlings are then exposed in the freezer room for a number of hours to a temperature at some point between -10° and -20°. The length of exposure to low temperatures is so gauged that about 50 per cent of the control alfalfa survives. After freezing, the seedlings are removed to the greenhouse and two weeks later survival counts are made. The actual percentages of survival of the alfalfas are calculated in terms of the control alfalfa, and comparisons between alfalfas are made by this standard.

This method gives reliable and consistent results in the determination of relative hardness in different alfalfas, and offers a rapid means whereby they can be tested for comparative cold resistance. This method may also be used for selecting harder types within a strain or variety of alfalfa.

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crossover percentage of  $28.70 \pm 3.43$  for the repulsion phase and  $34.54 \pm 2.89$  for the coupling phase

Buckley (1) reports a linkage of the factor pairs *Bl bl* for blue versus white aleurone and *Kk* for hoods versus awns. A minimum  $\chi^2$  value of 2.23 was obtained at a crossover value of 41 per cent.

Hallquist (4) reports a linkage of the following chlorophyll-defective seedling factors: Albino<sub>4</sub>, albino<sub>3</sub>, and chlorina. The crossover percentages were found to be 10.2 for albino<sub>3</sub> and chlorina, 3.8 for chlorina and albino<sub>4</sub>; and 12.5 for albino<sub>3</sub> and albino<sub>4</sub>.

#### VARIETIES USED IN THE EXPERIMENTS

This paper presents a study of the inheritance of various factor pairs and their possible linkage relations. The following varieties were used in the various studies: Coast C I No 2791, Lion C I. No 923, Minnesota 84-7, Trebi, Coast III, Colseess I, Colseess IV, Colseess V, and Minnesota 72-8.

Coast C I No 2791 has a white glume, short-haired rachilla, blue aleurone, branched styles, and rough awn. The barbing extends the full length of the awn. The grain is hulled.

Lion C I. No 923 has black glumes, long-haired rachilla, unbranched styles, and smooth awns. However, there is barbing of the awns at the base, which was disregarded in this study. Also, there is some barbing on the tip of the awns which varies, as is shown by the awn indices.

Minnesota 84-7 is a 2-rowed, white-hulled, awned barley. The rachilla hairs are long and it carries the factor pair *ff* for chlorina plant color. This barley was obtained from the Minnesota station and came originally from C. Hallquist. Nilsson-Ehle (9) describes it as a pale-green chlorophyll-deficient type. It is a 2-rowed strain of Gold. The color of the seedlings is "cosse green" (Ridgway, Pl. V) <sup>4</sup>. The plants grow to maturity but are somewhat stunted.

Trebi (10) is a 6-rowed, bearded, hulled barley with heads very similar to those of Coast. The strain Trebi I carries a factor pair (*A<sub>1</sub>a<sub>1</sub>*) for green versus white seedlings which has previously been found linked with the factor pair *Bb* for black versus white glume color.

Coast III is similar to Coast, but carries a factor pair (*Y<sub>2</sub>y<sub>2</sub>*) for green versus virescent seedlings (11). The virescent seedling dies in the seedling stage. It comes up with a very marked green tip on the first leaf.

Colseess is a 6-rowed hooded barley with a hulled grain of a bluish-green color. The straw and glume are light yellow. The shank of the hood is about 5 mm long and is barbed at the base. The rachis is rather tough and the head does not shatter easily. The rachilla hairs are short and the outer glume is covered with very short hairs. Several strains of this variety have been used in the studies of chlorophyll deficiencies. Colseess I and Colseess IV (10) carry the factor pair *A<sub>1</sub>a<sub>1</sub>* for green versus white seedlings and Colseess IV carries the factor pair *X<sub>1</sub>x<sub>1</sub>* for green versus xantha seedlings. The factor pairs *A<sub>1</sub>a<sub>1</sub>* and *X<sub>1</sub>x<sub>1</sub>* have previously been found to be closely linked. The strain Colseess V (11) is a chlorina plant, less vigorous than the normal

<sup>4</sup> RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., illus., Washington, D. C. 1912.

TABLE 1—Character pairs discussed in this paper which have been studied by previous workers—Continued

Character pair	Investigator	Number of factors involved	Symbol used
Green v. albino	Hallquist (4)	Single-factor, difference	( <i>lgl</i> )
Green v. albino	Robertson (10)	do	( <i>Ala</i> )
Green v. xantha	do	do	( <i>Xcr</i> )
Green v. albino	do	do	( <i>Ala</i> )
Do	Robertson and Deming (11)	do	( <i>lgl</i> )
Green v. yellow	do	do	( <i>XcrXcr</i> )
Green v. virescent	do	do	( <i>Ycl</i> )
Green v. chlorina	do	do	( <i>Fcl</i> )

Three types of  $F_2$  segregations have been described in differentiating between rough and smooth awns. Hayes and Garber (5), in summarizing previous data, report that the  $F_1$  plants in crosses between rough and smooth awned varieties have rough awns, while the  $F_2$  generation segregated into rough and smooth awned plants in an approximate 3:1 ratio. Griffiee (3) and Sigfusson (12) report a 2-factor difference. Griffiee was able to divide the  $F_2$  plants into three phenotypes only based on an arbitrary awn index division. The types rough, intermediate smooth, and smooth were obtained in a 12:3:1 ratio. The factor *R* produces rough awns, while the factor *S* is hypostatic to *R* and in the absence of *R* produces intermediate-smooth awns. The double recessive *rr ss* produces smooth awns. Sigfusson classifies the  $F_2$  plants into four groups according to the degree of roughness. He states (12, p. 666):

The rough and intermediate rough classes have barbs the entire length of the awn, but the awns of the latter class are not nearly as scabrous. When heads of these phenotypes were examined in sunlight, and when held at the correct distance from the eye to be properly focussed, the difference could be easily discerned.

Sigfusson classified the  $F_2$  as rough, intermediate rough, intermediate smooth, and smooth. A close approximation, of a 9:3:3:1 ratio was obtained. The factor *R*, either single or in duplicate and in the absence of *S*, produced the intermediate-rough condition, and likewise the factor *S*, in the absence of *R*, produced the intermediate-smooth condition. Both factors are necessary to produce the fully barbed condition. The double recessive *rr ss* produced the smooth-awn class.

Daane (2) reviews the previous work on linkage and describes five linkage groups. (1) The non 6-rowed versus 6-rowed character pair, (2) black versus white lemma and pericarp; (3) hulled versus naked seed; (4) hooded versus awned; and (5) rough versus smooth awn. Several other characters have been found which have not yet been placed in any of the linkage groups described.

Hor (7) reports a linkage between black versus white glume color, rough versus smooth awn, and long versus short haired rachilla.

Robertson (10), Sigfusson (12), and Buckley (1) found the factor pairs for black versus white glume color and rough versus smooth awn to be inherited independently.

Sigfusson (12) and Hor (7) obtained a linkage between long versus short haired rachilla and one of the factors for rough versus smooth awn. Sigfusson gives a crossover percentage of 30.8. Hor found a

## BLUE VERSUS WHITE ALEURONE (Bl Bl)

The inheritance of blue and white aleurone was studied in a cross between Colless IV and Minnesota 72-8. A separation of the blue and white aleurone color was attempted in the  $F_1$  plants, but some difficulty was encountered, especially with small immature seeds. The  $F_2$  plants, however, were separated in the field into homozygous blue aleurone, heterozygous plants having both blue and white seeds on the same head, and homozygous white aleurone. When the plants were grouped into two groups, those containing homozygous and heterozygous blue aleurone and those with colorless aleurone, the segregations shown in Table 4 were obtained.

TABLE 4— $F_2$  segregation of seeds with colored aleurone and colorless aleurone as determined from  $F_2$  plants, in Colless IV  $\times$  Minnesota 72-8

Item	Colored	Colorless	Deviation	D P E
Observed count.....	4,553	1,395		
Calculated segregation, 3 : 1.....	4,461	1,487	92	4.03

The number of plants with colorless aleurone is small. When plants are grouped as homozygous blue and heterozygous blue and colorless, a much better fit to the calculated 3 : 1 ratio is obtained. Table 5 gives the grouping of the different  $F_2$  seeds.

TABLE 5— $F_2$  segregation of seeds with pure blue aleurone (Bl Bl) and nonpure blue aleurone (Bl bl and bl bl) as determined from the  $F_2$  plants in Colless IV  $\times$  Minnesota 72-8

Item	Heterozygous blue and colorless	Homozygous	Deviation	D P E
Observed count.....	4,420	1,525		
Calculated segregation, 3 : 1.....	4,461	1,487	41	1.42

The results shown in Tables 4 and 5 may be explained as due to a single-factor pair.

The better fit in the classification of nonblue and blue-seeded plants may be explained by the fact that white seeds on  $F_2$  plants may have been fertilized with pollen carrying the factor for blue, and all plants showing any blue aleurone in the heads were classified as heterozygous. The progeny of some  $F_2$  white seeds ( $F_1$  plants) evidently were classified as heterozygous instead of colorless, which reduced the number of colorless plants.

## ROUGH VERSUS SMOOTH AWN

The inheritance of rough versus smooth awns was studied in a Coast  $\times$  Lion cross. The  $F_2$  plants were classified into three groups, rough, intermediate smooth, and smooth. The method used was the same as that employed by Hayes et al (6) and Griffiee (3). An awn of average length was taken from the center of the main spike of each plant and examined under the microscope. Hayes et al (6) state:

The distance on the tip of the awn upon which teeth were regularly borne was measured. The total length of the awn was divided by the length of the tip upon which teeth were found. The result obtained was called the awn index; the larger the index, the smoother the awn and vice versa.

green plants, and lighter in color. The plants are "dull green-yellow" (Ridgway, Plate XVII)<sup>5</sup>

Minnesota 72-8 is a 6-rowed, hulled, awned barley, obtained from the Minnesota station. The original plant came from Hallquist and has the factor pair  $Yy$  for green versus virescent seedlings. The virescent seedlings have a slightly green tip, but fail to survive beyond the seedling stage.

Methods similar to those used in previous work by the senior author(10) were employed.

The strains containing the chlorina factor pairs  $Ff$  and  $F_c f_c$  were recessive for the factors for chlorina. These chlorina plants were used as parents instead of plants heterozygous for the chlorophyll deficiencies used in previous studies.

## INHERITANCE OF SIMPLE MENDELIAN CHARACTERS

### CHLORINA SEEDLINGS IN MINNESOTA 84-7

The inheritance of the chlorina seedlings in Minnesota 84-7 was studied in a cross between chlorina plants of Minnesota 84-7 and Trebi plants heterozygous for the factor pair for green versus white seedlings  $A_1 a_1$ . All of the  $F_1$  plants were green, indicating that the factor pairs  $Ff$  and  $A_1 a_1$  are not allelomorphic. There were 9  $F_1$  plants which segregated in  $F_2$  for green and chlorina plants and 14 which segregated for green, chlorina and white. The segregation of the green and chlorina plants indicates a single-factor difference. Table 2 presents the data obtained from these crosses.

TABLE 2— $F_2$  segregation of green and chlorina seedlings in Minnesota 84-7  $\times$  Trebi

Item	Green	Chlorina	Deviation	D/PE
Observed count.....	1,453	512		
Calculated segregation, 3 : 1.....	1,473.75	491.25	20.75	1.6

### VIRESCENT SEEDLINGS IN MINNESOTA 72-8

The mode of inheritance of green and virescent seedlings was studied in a cross between Colless IV, heterozygous for the factor pair  $X_c x_c$  for green versus xantha seedlings and Minnesota 72-8, heterozygous for the factor pair  $Yy$  for green versus virescent seedlings. The number of green and virescent seedlings obtained from the progeny of six  $F_1$  plants which were heterozygous for the seedling factor pair  $Yy$  is shown in Table 3.

TABLE 3— $F_2$  segregation of green and virescent seedlings from plants heterozygous for the green and virescent factor pair ( $Yy$ ) in Colless IV  $\times$  Minnesota 72-8

Item	Green	Virescent	Deviation	D/PE
Observed count.....	926	327		
Calculated segregation, 3 : 1.....	939.75	313.25	13.75	1.33

The segregation of green and virescent seedlings indicates a single-factor difference.

<sup>5</sup> RIDGWAY, R. Op cit

the factors to be recessive in order to produce the unbranched styles of the *Leon* type.

### INTERRELATIONSHIP OF CHLOROPHYLL DEFICIENCIES

#### GREEN VERSUS CHLORINA (*Ff*) AND GREEN VERSUS WHITE SEEDLINGS (*A<sub>1</sub>a<sub>1</sub>*)

The interrelationship of the factor pairs *Ff* and *A<sub>1</sub>a<sub>1</sub>* was studied in a cross between Minnesota 84-7 homozygous for chlorina (*ff*) and Trebi, heterozygous for green and white seedlings (*A<sub>1</sub>a<sub>1</sub>*). All the *F<sub>1</sub>* plants were green, indicating that the factor pairs *Ff* and *A<sub>1</sub>a<sub>1</sub>* are not allelomorphic. There were 9 *F<sub>1</sub>* plants that segregated in *F<sub>2</sub>* for green and chlorina and 14 that segregated for green, chlorina, and white. The interrelationship of the factor pairs *Ff* and *A<sub>1</sub>a<sub>1</sub>* was studied in the progeny of the 14 plants that segregated for green, chlorina, and white seedlings (Table 7)

TABLE 7—*F<sub>2</sub>* segregation of green, chlorina, and white seedlings in Minnesota 84-7 × Trebi

Item	Green	Chlorina	White
Observed count.....	3,333	1,164	1,554
Calculated segregation, 9:3:4.....	3,403.7	1,134.6	1,512.7

$$\chi^2 = 3.3579. \quad P = 0.1917$$

The test for goodness of fit to a 9:3:4 ratio gave a *P* value of 0.1917, indicating independent inheritance of the factor pairs *A<sub>1</sub>a<sub>1</sub>* and *Ff*.

#### GREEN VERSUS CHLORINA (*Ff*) AND GREEN VERSUS WHITE SEEDLINGS (*A<sub>2</sub>a<sub>2</sub>*)

The inheritance of green versus chlorina (*Ff*) and green versus white seedlings (*A<sub>2</sub>a<sub>2</sub>*) was studied in a cross between Minnesota 84-7 and Colseß. The following crosses were grown in *F<sub>1</sub>* and *F<sub>2</sub>*: II-26-440, II-26-421, II-27-148, and II-27-151. All the *F<sub>1</sub>* plants were green, indicating that the factor pair for green versus chlorina (*Ff*) and the factor pair for green versus white in Colseß (*A<sub>2</sub>a<sub>2</sub>*) are not allelomorphs. From a cross of the above types, where a pure chlorina plant *ff* is crossed with a plant heterozygous for green and white seedlings *A<sub>2</sub>a<sub>2</sub>*, only two kinds of *F<sub>1</sub>* plants would be expected, namely, those giving progeny segregating in *F<sub>2</sub>* into green and chlorina seedlings and those segregating into green, chlorina, and white seedlings. The progeny of 10 plants of the former type and 7 of the latter were studied.

The interrelationship of the factor pairs *Ff* for green versus chlorina seedlings and *A<sub>2</sub>a<sub>2</sub>* for green versus white seedlings was studied in the families that segregated for all three types of seedlings. The following segregations were obtained: Crosses II-27-148 and II-27-151 produced 1,397 green, 541 chlorina, and 599 white seedlings. The number of chlorina plants is greater than the calculated 9:3:4 ratio. A similar but wider variation was found in cross II-26-421. The *F<sub>2</sub>* plants segregated into 776 green, 302 chlorina, and 308 white. In order to test the possibility of linkage, the *F<sub>3</sub>* segregation from the *F<sub>2</sub>* green plants was determined (Table 8)

Coast and the rough  $F_2$  plants have an awn index of 1. The awn indices of 100 plants of the Lion parent were determined. The awn indices of this group ranged from 2.7 to 7.6, with an average of 4.3. A division of the partially smooth plants was made at the awn index of 2.7, which was the lower limit of the Lion plants. The plants with an awn index of 2.7 or more were classified as smooth, and the plants with an awn index of 1.1 to and including 2.6 were classified as intermediate smooth.

As shown in Table 6, the observed segregation approaches a 12:3:1 ratio. By applying the test for goodness of fit, a  $\chi^2$  value of 2.28 was obtained with a  $P$  value of 0.3273. This segregation is similar to that found by Griffie (3), and can best be explained on the basis of 2-factor difference for roughness of awn.  $R$  and  $R'$  are used to designate the factors for roughness of awn, and  $r$  and  $r'$  denote the absence of the rough condition. When the factor  $R$  is present the awn is rough.  $R'$  is hypostatic to  $R$ , and in the absence of  $R$  gives intermediate smooth awned plants. The double recessive,  $rr r' r'$ , gives the smooth group similar to the Lion parent.

TABLE 6—Segregation in the  $F_2$  generation for rough, intermediate smooth, and smooth awns in  $Coast \times Lion$

Item	Rough <sup>a</sup>	Inter- mediate smooth <sup>a</sup>	Smooth <sup>a</sup>
Observed count.....	852	141	61
Calculated segregation, 12 3 1.....	830 25	207 56	69 19

$\chi^2=2.28$   $P=0.3273$

<sup>a</sup> Awn indices: Rough, 1, intermediate smooth, 1.1 to 2.6, smooth, 2.7 or over

#### BRANCHING OF STYLE

In a previous paper, Robertson and Deming (11), have reported a 3-factor difference between the smooth style of Lion and the feathered style of Coast. The factor pairs are designated  $Gg G'g' G''g''$ . The behavior of about 50  $F_3$  plants from each  $F_2$  family was studied. With independent inheritance of three cumulative factors, the following ratio of differently segregating plants would be expected in the  $F_3$  generation. Thirty-seven plants would have one or more of the factors in the homozygous dominant condition and would give plants with some degree of branching on the style, 12 plants would segregate 15 branched to 1 unbranched, 8 would segregate 63 branched to 1 unbranched, 6 would segregate 3 branched to 1 unbranched, and 1 would breed true for the unbranched condition.

The following data were obtained from the  $F_3$  families studied. Forty-five  $F_3$  families segregated 15:1, 35 segregated 3:1, 17 segregated 63:1, and all of the  $F_2$  plants with unbranched styles bred true. A poor fit to the calculated number of segregating families was obtained. Some error evidently crept in from the small number of plants in the  $F_3$  families, and possibly this would account for the small number of families segregating 63:1. However, when we consider the close fit of the  $F_2$  segregation to the calculated 63:1 ratio and the fact that all the calculated  $F_3$  ratios were obtained, it may be concluded that these results are best explained on a 3-factor hypothesis. These factors are cumulative in their effect, and it is necessary for all

In order further to test the linkage relationship, the  $F_2$  genotypes of the green plants in the  $F_2$  families segregating for green, chlorina, and virescent seedlings were determined from  $F_3$  seedling counts. Table 11 gives the grouping of 746  $F_2$  plants in the different genotypes, as determined by  $F_3$  seedling counts. As in the  $F_2$  data, the observed to the calculated 1:2:2:4 ratio for independent inheritance gives a very poor fit.

TABLE 11—Grouping of 746  $F_2$  green plants in the different genotypes as determined by  $F_3$  seedling counts of their progeny

Item	Number of plants showing indicated breeding habit in $F_3$			
	Pure green	Green chlorina	Green virescent	Green chlorina virescent
Ratio.....	(*)	3 1	3 1	9 3 4
Observed counts.....	31	145	157	413
Calculated segregation, 1 2 2 4.....	82 9	165 8	165 8	331 5

$\chi^2=55.6058$   $P$ , very small

\* All

The possibility of linkage was calculated from the formula used by Robertson (10) and a crossover value of 29.30 was obtained. The observed ratio was compared with the calculated ratio with 29.30 per cent crossing over. Table 12 gives the results obtained when the  $\chi^2$  test for goodness of fit is used.

TABLE 12—Observed and calculated  $F_2$  genotypes of 746  $F_2$  plants obtained from Coast III  $\times$  Colseess V

Item	$F_2$ genotypes of indicated breeding habit			
	Green	Green chlorina	Green virescent	Green chlorina virescent
Observed count.....	31	145	157	413
Calculated segregation, 29.3 per cent crossing over.....	30 7	148 2	148 2	418 9

$\chi^2=0.6777$   $P$ , very large

The fit of the observed to the calculated ratio with 29.30 per cent crossing over is very good, indicating a linkage of the factor pairs  $Ycy_c$  for green versus virescent seedlings in Coast III and the factor pairs  $F_cfc_c$  for green versus chlorina seedlings in Colseess V. The chlorina factor pair  $f_cfc_c$  had previously been shown by two of the writers (11) to be inherited independently of the factor pairs  $A_cac_c$  and  $Xcx_c$  found in Colseess and the factor pair  $A_cac_c$  for green versus white seedlings found in Trebi. The factor pair  $Kk$  for hoods versus awns was also found to be inherited independently of the chlorina factor pair. The factor pair  $Ycy_c$  for green versus virescent seedlings in Coast was also reported to be inherited independently of the factor pairs  $A_cac_c$ ,  $Xcx_c$ ,  $A_cac_c$ , and  $Kk$ .

TABLE 8—*Genotypes of F<sub>2</sub> green plants as determined from F<sub>3</sub> seedling counts in various crosses*

Cross No	Number of plants having indicated genotype					
	<i>FFA<sub>c</sub>A<sub>c</sub></i>	<i>Ff A<sub>c</sub> A<sub>c</sub></i>	<i>FFA<sub>c</sub>a<sub>c</sub></i>	<i>FfA<sub>c</sub>a<sub>c</sub></i>	$\chi^2$	<i>P</i>
II-26-440	76	170	152	331	1 47.22	0 69.32
II-26-421	35	80	79	171	1 64.76	65.30
II-27-148 and 151	133	265	218	522	6 25.35	1015

The data in Table 8 indicate independent inheritance of the factor pairs *Ff* for green versus chlorina seedlings and *A<sub>c</sub>a<sub>c</sub>* for green versus white seedlings

GREEN VERSUS VIRESCENT SEEDLINGS *Y<sub>c</sub>y<sub>c</sub>* AND GREEN VERSUS CHLORINA SEEDLINGS *F<sub>c</sub>f<sub>c</sub>*

The relationship of the factor pairs concerned was studied in a cross between Coast, heterozygous for green and virescent seedlings *Y<sub>c</sub>y<sub>c</sub>* and Colsees, homozygous for chlorina seedlings *f<sub>c</sub>f<sub>c</sub>*.

Two types of segregating progeny were obtained. Eleven *F<sub>1</sub>* plants segregated for green and chlorina seedlings in *F<sub>2</sub>*, and 22 *F<sub>1</sub>* plants segregated for green, chlorina, and virescent seedlings in *F<sub>2</sub>*. The observed ratio as compared with a calculated 9:3:4 ratio is shown in Table 9. The data here given indicate a very poor fit to the calculated 9:3:4 ratio.

TABLE 9.—*Observed and calculated 9:3:4 ratio of green, chlorina, and virescent seedlings in the F<sub>2</sub> generation from Coast III × Colsees V*

Item	Green	Chlorina	Virescent
Observed count	4,165	1,823	1,853
Calculated segregation, 9:3:4	4,410.5	1,470.2	1,960.3

$\chi^2=104.987$  *P*, very small

In order to determine whether linkage was present, Collins's formula

$$p = \sqrt{\frac{AB - 2Ab}{AB + Ab}}$$

was used. A crossover percentage of 29.44 was obtained. The observed and calculated ratio on the basis of 29.44 crossing over is given in Table 10. The data here shown indicate that there is a possible linkage between the factor pairs *Y<sub>c</sub>y<sub>c</sub>* and *F<sub>c</sub>f<sub>c</sub>*. While the *P* value is low, it shows a better fit than that obtained when the observed ratio is tested with a 9:3:4 ratio.

TABLE 10.—*Observed and calculated segregation with 29.44 per cent crossing over in Coast III × Colsees V*

Item	Green	Chlorina	Virescent
Observed count	4,165	1,823	1,853
Calculated segregation, 29.44 per cent crossing over	4,090.5	1,790.3	1,960.2

$\chi^2=7.8165$  *P*=0.0205

## INTERRELATIONSHIP OF THE YELLOW AND VIRESCENT SEEDLING FACTORS

The progeny of  $F_1$  green plants which segregated for all three types of seedlings in  $F_2$  were used. In Table 15 the plants are grouped according to the  $F_2$  genotype, as determined from the  $F_3$  seedling segregations

TABLE 15 —Observed and calculated  $F_2$  genotypes, as determined by the  $F_3$  seedling segregations in Colless IV  $\times$  Minnesota 72-8

Item	Number of indicated genotype			
	$X_cX_cYY$	$X_cX_cYy$	$X_cX_cYY$	$X_cX_cYy$
Observed count.....	171	352	315	646
Calculated segregation, 1 2 2 4.....	164 9	329 8	329 8	659 6

$$\chi^2=2.6647 \quad P=0.4522$$

The data in Table 15 indicate that the factor pairs  $X_cX_c$  and  $Yy$  are inherited independently of each other

## RELATION OF CHLOROPHYLL DEFICIENCIES TO OTHER BOTANICAL CHARACTERS

GREEN VERSUS CHLORINA SEEDLINGS ( $Ff$ ) AND LONG VERSUS SHORT HAIRED RACHILLA ( $Ss$ )

The interrelationship of the green versus chlorina seedling color and long versus short haired rachilla was studied in the  $F_2$  plants producing only green and chlorina seedlings. Table 16 gives the observed values and the calculated 9:3:3:1 ratio for green versus chlorina seedlings and long versus short haired rachilla

TABLE 16 — $F_2$  segregation of green versus chlorina seedlings and long versus short haired rachilla

Item	Number of plants having character indicated			
	Green		Chlorina	
	Long	Short	Long	Short
Observed count.....	810	266	275	95
Calculated segregation, 9 3 3 1.....	813 4	271 1	271 1	90 4

$$\chi^2=0.4029 \quad P, \text{ very large}$$

The agreement between the observed and the calculated ratio for independent inheritance is very good and indicates that the factor pairs  $Ff$  and  $Ss$  are inherited independently of each other.

GREEN VERSUS CHLORINA SEEDLINGS ( $Ff$ ) AND NON 6-ROWED VERSUS 6-ROWED ( $Vv$ )

In studying the interrelationship of the non 6-rowed and 6-rowed character pair with green versus chlorina, it was found that the green plants were high in 6-rowed plants and the chlorina plants were high in non 6-rowed plants. The 6-rowed plants had the following genotypes,  $vvII$ ,  $vvIi$ , and  $vvii$ . If the non 6-rowed plants are grouped as

GREEN VERSUS CHLORINA SEEDLINGS ( $Ff$ ) IN MINNESOTA 84-7 AND GREEN VERSUS CHLORINA SEEDLINGS ( $F_c f_c$ ) IN COLSESS

The interrelationship of the factor pairs  $Ff$  and  $F_c f_c$  was studied in a cross between Minnesota 84-7 pure for chlorina ( $ff$ ) and a Colseess plant pure for chlorina ( $f_c f_c$ ). The  $F_1$  plants were pure green. The  $F_2$  plants segregated into green and chlorina. Table 13 gives the data obtained for several  $F_2$  families. The chlorina plants were hard to separate in the field and were grouped together.

TABLE 13— $F_2$  segregation of green and chlorina plants from Minnesota 84-7  $\times$  Colseess V

Item	Green	Chlorina	D/P/E
Observed count.....	4,485	3,697	
Calculated segregation, 9:7.....	4,602	3,580	3:91

The number of chlorina plants is larger than the calculated number, the deviation divided by the probable error is 3.91.

The segregation of the green and the two chlorina types was determined from  $F_3$  seedling counts. Table 14 gives the  $F_3$  segregation of green and the different chlorinas, as determined from the  $F_3$  seedling counts. The data here given indicate that the factor pair  $Ff$  for green and chlorina seedlings in Minnesota 84-7 and the factor pair  $F_c f_c$  for green and chlorina in Colseess V are inherited independently of each other. To test further the inheritance of the factor pairs  $Ff$  and  $F_c f_c$ ,  $F_3$  seedling counts were made on the progeny of  $F_2$  green plants. With independent inheritance, a ratio of 1 green to 2 segregating for green and chlorina,  $Ff$ , 2 segregating for green and chlorina,  $F_c f_c$ ; and 4 segregating for green and both chlorinas would be expected. A close approach to a calculated 1:2:2:4 ratio was obtained,  $\chi^2 = 1.9345$ , which gave a  $P$  value of 0.5874. This further indicated that the factor pairs  $Ff$  and  $F_c f_c$  are inherited independently of each other.

TABLE 14— $F_3$  classes as determined from  $F_2$  seedling counts in Minnesota 84-7  $\times$  Colseess V

Item	Green	Minnesota 84-7 chlorina	Colseess V chlorina	Double chlorina
Observed count.....	1,158	346	322	120
Calculated segregation for independence.....	1,143.8	360.2	336.2	105.8

$$\chi^2 = 3.2418 \quad P = 0.0718$$

GREEN VERSUS XANTHA SEEDLINGS ( $X_c x_c$ ) AND GREEN VERSUS VIRESCENT SEEDLINGS ( $Yy$ )

Crosses were made between Colseess IV plants heterozygous for yellow seedlings ( $X_c x_c$ ) and Minnesota 72-8 heterozygous for virescent seedlings ( $Yy$ ) (green-tipped whites). Twenty-nine  $F_1$  plants were grown. Of these, 11 gave only pure-green progeny, 6 segregated for green and yellow, 6 segregated for green and virescent seedlings, and 6 segregated for green, yellow, and virescent seedlings. The number of green plants was somewhat larger than expected.

Table 18 indicates a linkage of the factor pairs *Ff* and *Vv*, with a crossover value of 18.3 per cent. The probable error for the crossover value is  $\pm 0.74$  per cent.

When the non 6-rowed versus the 6-rowed and green versus chlorina plants are tested, there is again a poor fit of the observed to the calculated 9:3:3:1 ratio. The segregation of this type is opposite to the previous classification for rows and is in the repulsion phase, since non 6-rowed and chlorina went into the cross together and 6-rowed and green went into the cross together. The crossover value was again determined by the product-moment method, and a crossover percentage of  $16.76 \pm 1.65$  per cent was obtained. Table 19 presents the  $F_2$  observed ratio and the calculated ratio with 16.76 per cent crossover. The fit of the observed to the calculated ratio is good in both cases.

TABLE 19— $F_2$  segregation of non 6-rowed versus 6-rowed, and green versus chlorina plants compared with a calculated  $F_2$  ratio with 16.76 per cent crossing over

Item	Number of plants having characters indicated			
	Green		Chlorina	
	Non 6-rowed	6-rowed	Non 6-rowed	6-rowed
Observed count.....	783	363	393	11
Calculated segregation 16.76 per cent crossing over.....	785.8	376.6	376.6	10.9

$$\chi^2 = 1.1318 \quad P = 0.7711$$

From the data presented in Tables 18 and 19, it may be concluded that the factor pair *Ff* for green versus chlorina seedlings is closely linked to the factor pair *Vv*, which distinguishes the characters 2-rowed and 6-rowed. The crossover value is about  $18.3 \pm 0.74$  per cent.

#### GREEN VERSUS CHLORINA (*Ff*) AND HOODS VERSUS AWNS (*Kk*)

The interrelationship of the green versus chlorina factor pair, *Ff*, and hoods versus awns, *Kk*, was studied in a cross between Colseess and Minnesota 84-7. The  $F_2$  segregation of the green versus chlorina plants for hoods versus awns is given in Table 20. The observed ratio fits the calculated 9:3:3:1 ratio very well,  $\chi^2$  being 2.5725 with a *P* value of 0.4689. This indicates that the factor pairs *Ff* and *Kk* are inherited independently of each other.

TABLE 20—Segregation of green versus chlorina (*Ff*) plants and hoods versus awns (*Kk*) in Colseess  $\times$  Minnesota 84-7

Item	Number of plants having characters indicated			
	Green		Chlorina	
	Hooded	Awned	Hooded	Awned
Observed count.....	931	326	326	119
Calculated segregation, 9:3:3:1.....	957.4	319.1	319.1	106.4

$$\chi^2 = 2.5725 \quad P = 0.4689$$

2-rowed and intermediate plants, the 2-rowed plants would have the following genotypes, *VVII*, *VViI*, and *VVv*. This type of classification can be made in the  $F_2$ . If the genotypes of the intermediate classes *VvI*, *Vvn*, *VvII* are grouped the ratio is as follows. One 6-rowed, two intermediate, and one 2-rowed. The intermediate classes contained both high and low fertility types of intermediates. A check of the  $F_2$  counts was made from  $F_3$  segregation of  $F_2$  plants. Only four changes in classification if 619  $F_2$  plants were necessary. Two 2-rowed plants were changed to an intermedium, one intermediate high-fertility plant to a 6-rowed, and one intermediate to an intermedium. This would only change the place of two plants in the 1:2:1 ratio.

When 2-rowed plants (*VVII*, *VViI*, and *VVv*) are grouped in one class and the non 2-rowed (6-rowed *vvII*, *vvI*, *vvn*, and intermediate *VvI*, *Vvn*, *VvII*) are grouped in the other class, a ratio approaching 1 2-rowed to 3 non 2-rowed was obtained. Similarly, when the  $F_2$  plants were classified as non 6-rowed (2-rowed *VVII*, *VViI*, *VVv*, and intermediate *VvI*, *Vvn*, and *VvII*) and 6-rowed (*vvII*, *vvI*, and *vvn*) a good fit to a 3:1 ratio was obtained.

When the interrelationship of green versus chlorina and non 2-rowed versus 2-rowed is studied, a coupling type of linkage is found. Table 17 gives the  $F_2$  segregation of non 2-rowed versus 2-rowed and green versus chlorina plants. The fit of the observed to the calculated is very poor.

TABLE 17 —  $F_2$  segregation of non 2-rowed versus 2-rowed and green versus chlorina plants

Item	Number of plants having characters indicated			
	Green		Chlorina	
	Non 2-rowed	2-rowed	Non 2-rowed	2-rowed
Observed count.....	1,028	118	142	262
Calculated segregation, 9 3 3 1.....	871 9	290 6	290 6	96 9

$\chi^2 = 487.75$   $P$ , very small

The product-moment method was used to determine the crossover percentage between the factor pairs *Vv* and *Fy*. Immer's (8) tables were used. A crossover percentage of 18.3 was obtained. Table 18 gives the fit of the observed to the calculated ratio with 18.3 per cent of crossing over.

TABLE 18 —  $F_2$  segregation of non 2-rowed versus 2-rowed and green versus chlorina plants compared with a calculated ratio with 18.3 per cent crossing over

Item	Number of plants having characters indicated			
	Green		Chlorina	
	Non 2-rowed	2-rowed	Non 2-rowed	2-rowed
Observed count.....	1,038	118	142	262
Calculated segregation, 18.3 per cent crossover.....	1,033 6	128 9	128 9	268 6

$\chi^2 = 2.3280$   $P = 0.5131$

TABLE 23—Observed and calculated 9 3 3 1 ratio of nonblue versus blue and hoods versus awns in Colless  $\times$  Minnesota 72-8

Item	Number of plants having characters indicated			
	Nonblue		Blue	
	Hooded	Awned	Hooded	Awned
Observed count.....	3,086	1,334	1,455	73
Calculated segregation, 9 3 3 1.....	3,345 75	1,115 25	1,115 25	371 75

 $\chi^2=406.65$   $P$ , very small

The observed ratio fits the calculated 9:3:3:1 ratio very poorly. The two middle classes, however, are noticeably high and the two extreme classes low. Since nonblue went into the cross with awns, and blue went into the cross with hoods, a linkage of the repulsion type might be expected. The segregation of the  $F_2$  plants indicates such a linkage. The possible linkage value was calculated by the product-moment method with the use of Immer's tables (8), and a crossover value of  $22.58 \pm 0.82$  per cent was obtained.

The data in Table 24 indicate a linkage of the factor pairs  $Bl\ bl$  and  $Kk$ . The linkage agrees with the finding of Buckley (1). However, he found a crossover value of 40.56. In his studies he used 714 plants

TABLE 24—Observed and calculated ratio of nonblue versus blue and hoods versus awns with 22.58 per cent crossing over

Item	Number of plants having character indicated			
	Nonblue		Blue	
	K	k	K	k
Observed count.....	3,086	1,334	1,455	73
Calculated segregation, 22.58 per cent crossing over.....	3,049 8	1,411 2	1,411 2	75 8

 $\chi^2=6.1157$   $P=0.1070$ 

Similar wide deviations from the calculated 9:3:3:1 ratio were found for the factor pairs  $Bl\ bl$  and  $Kk$  in the families segregating for green and yellow seedlings in the  $F_2$  and also in the families segregating for green and virescent seedlings in the  $F_2$ . As has already been shown, the nonblue and blue segregation of aleurone color both gave good fits to the calculated 3:1 ratio. There is evidently no linkage between the factor pairs  $X_c x_c$  for green versus yellow seedlings and  $Y y$  for green versus virescent seedlings and aleurone color.

Crossover percentages were calculated for the factor pairs  $Bl\ bl$  and  $Kk$  in families segregating for green and yellow seedlings and families segregating for green and virescent seedlings. The crossover values were  $25.01 \pm 1.19$  and  $24.70 \pm 1.21$ . In the former case 2,760 plants were used and in the latter 2,667.

When the observed and calculated values with 25.01 and 24.70 crossover percentage were tested, a  $\chi^2$  of 1.5606 with a  $P$  value of 0.6602 was obtained in the families segregating for green and yellow

GREEN VERSUS XANTHA SEEDLINGS ( $X_c x_c$ ) AND NONBLUE VERSUS BLUE ALEURONE ( $Bl bl$ )

The relationship of the factor pair for green versus xantha seedlings ( $X_c x_c$ ) and the factor pair for nonblue versus blue aleurone ( $Bl bl$ ) was tested in a cross between Colseess IV and Minnesota 72-8. There was no indication of a discrepancy in the 3:1 ratio of plants with nonblue to those with blue aleurone, as is shown in Table 21.

TABLE 21.— $F_2$  segregation of plants with nonblue and blue aleurone in families which produced xantha seedlings in Colseess IV  $\times$  Minnesota 72-8

Item	Nonblue	Blue	D/PE
Observed count.....	2,056	704	
Calculated segregation, 3:1.....	2,070	690	0.91

With close linkage of the blue factor and xantha seedlings, there should be a smaller number of plants with blue aleurone, since the factor  $Bl$  and  $x_c$  went into the cross together. No such condition is found, indicating independence of the factor pairs  $Blbl$  and  $X_c x_c$ .

GREEN VERSUS CHLORINA SEEDLINGS ( $F_c f_c$ ) AND LONG VERSUS SHORT HAIR ED RACHILLAS ( $Ss$ )

The interrelationship of green versus chlorina seedlings  $F_c f_c$  and long versus short haired rachilla  $Ss$  was studied in a cross between Colseess V and Nepal. The  $F_2$  segregation is given in Table 22. The data in this table indicate that the factor pairs  $F_c f_c$  and  $Ss$  are inherited independently of each other.

TABLE 22.— $F_2$  segregation of green versus chlorina ( $F_c f_c$ ) seedlings and long versus short haired rachillas ( $Ss$ ) in Colseess V  $\times$  Nepal

Item	Number of plants having character indicated			
	Green		Chlorina	
	S	s	S	s
Observed count.....	1,392	486	387	119
Calculated segregation for independence.....	1,401.4	478.6	377.6	128.4

$$\chi^2 = 1.1707 \quad P = 0.6547$$

## INTERRELATIONSHIP OF OTHER BOTANICAL CHARACTERS

BLUE VERSUS NONBLUE ALEURONE ( $Bl bl$ ) AND HOODS VERSUS AWNS ( $Kk$ )

The interrelationship of the factor pairs  $Bl bl$  and  $Kk$  was studied in a cross between Colseess and Minnesota 72-8. The Colseess seeds have a blue aleurone and Minnesota 72-8 a white aleurone. The aleurone color was separated into nonblue and blue, as previously mentioned. Table 23 gives the segregation of nonblue versus blue and hoods versus awns.

BLACK VERSUS WHITE GLUMES (Bb) AND BRANCHED VERSUS UNBRANCHED STYLES  
(Gg G'g' G''g'')

The interrelationship of the characters glume color and style branching was studied in a (63 1) (3 1) classification of  $F_2$  data. Table 27 presents the results obtained.

TABLE 27— $F_2$  segregation of style branching (63 1) and glume color (3 1) in Coast x Lion

Item	Number of plants having characters indicated			
	Branched style		Unbranched style	
	Black	White	Black	White
Observed count.....	806	282	16	3
Calculated segregation (63 1) (3 1).....	816	272	14 25	4 75

$$\chi^2=1.35 \quad P=0.520$$

The data indicate independent inheritance of the factor pairs Gg G'g' G''g'' and the factor pair Bb.

LONG VERSUS SHORT HAIRED RACHILLA (Ss) AND ROUGH VERSUS SMOOTH AWN  
(Rr R'r')

In studying the interrelationship of long versus short haired rachilla Ss and rough versus smooth awn, the factors for the rough-awned character were studied separately. Two types of classification of  $F_2$  material were made. One separated the material into rough and smooth. The smooth class included both intermediate smooth and smooth. Table 28 presents the data obtained from the two types of classification. When the  $\chi^2$  test for independence was used a very poor fit was obtained.

TABLE 28— $F_2$  segregation of long versus short haired rachillas and rough versus smooth awns

Item	Number of plants having characters indicated			
	Long-haired rachillas		Short-haired rachillas	
	Rough awns	Smooth awns	Rough awns	Smooth awns
Observed count.....	593	223	259	32
Calculated segregation.....	628	188	224	67

$$\chi^2=32.2190 \quad P, \text{ very small}$$

In order to determine whether the factor pair R'r' was inherited independently of the factor pair Ss, which differentiates between long and short haired rachilla, a second type of classification was made in which only the intermediate-smooth and smooth-awned plants were used. The data obtained are given in Table 29.

seedlings, and a  $\chi^2$  of 0.5967 with a very large value for  $P$  was obtained in the families segregating for green and virescent seedlings. While the latter crossover values are slightly higher than that calculated from the green plants, the difference is within three times the probable error of a difference. The crossover percentage is evidently about 22.00.

BLUE VERSUS NONBLUE ALEURONE ( $Bb$ ) AND LONG VERSUS SHORT HAired RACHILLA ( $Ss$ )

A total of 2,555  $F_2$  plants was used in this study. A slight deviation in the nonblue and blue segregation was found. The blue-seeded plants were in larger numbers than the calculated 3:1 ratio of nonblue versus blue. In order to overcome the error caused by this discrepancy, the  $F_2$  segregation was tested for independence. The results given in Table 25 were obtained.

TABLE 25— $F_2$  segregation for nonblue versus blue and long versus short haired rachilla in pure green plants

Item	Number of plants having characters indicated			
	Nonblue		Blue	
	Long-haired rachillas	Short-haired rachillas	Long-haired rachillas	Short-haired rachillas
Observed count.....	1,386	471	511	187
Calculated segregation.....	1,378.75	478.25	518.25	179.75

$\chi^2=0.5418$   $P$ , very large

These data indicate that the factor pairs  $Bb$  and  $Ss$  are inherited independently of each other. This, again, agrees with Buckley's findings.

BLACK VERSUS WHITE GLUMES ( $Bb$ ) AND ROUGH VERSUS SMOOTH AWNS ( $Rr$   $R'r'$ )

The interrelationship of the factor pairs  $Bb$  and  $Rr$   $R'r'$  was studied in a Coast  $\times$  Lion cross. Table 26 presents the data obtained in this cross. The calculations were made on a (3 1) (12 3 1) basis. The data indicate that the factor pairs for rough versus smooth awns are inherited independently of the factor pair for black versus white glume color.

TABLE 26— $F_2$  segregation for glume color ( $Bb$ ) and roughness of awn ( $Rr$   $R'r'$ ) in Coast  $\times$  Lion

Item	Number of plants having characters indicated					
	Black Glumes			White Glumes		
	Rough	Intermediate smooth	Smooth	Rough	Intermediate smooth	Smooth
Observed count.....	635	138	49	217	51	15
Calculated segregation (3 1) (12 3 1).....	616.5	154.1	51.4	213.8	53.4	17.8

$\chi^2=2.85$   $P=0.5945$

age agrees fairly well with the crossover percentage of 30.8 reported by Sigfusson (12) and of  $28.70 \pm 3.43$  in the repulsion phase and  $34.54 \pm 2.89$  in the coupling phase reported by Hor (7)

ROUGH VERSUS SMOOTH AWN ( $Rr R'r'$ ) AND BRANCHED VERSUS UNBRANCHED STYLES ( $Gg G'g' G''g''$ )

The interrelationship of the factor pairs  $Rr R'r'$  for rough versus smooth awn and  $Gg G'g' G''g''$  for branched versus unbranched style was studied in the same cross. Table 31 gives the segregation of rough, intermediate-smooth, and smooth-awned plants with branched and unbranched styles.

TABLE 31— $F_2$  segregation of branched versus unbranched styles ( $Gg G'g' G''g''$ ) and rough versus smooth awns ( $Rr R'r'$ ) in *Coast*  $\times$  *Lion*

Item	Number of plants having characters indicated					
	Branched styles			Unbranched styles		
	Rough awns	Intermediate-smooth awns	Smooth awns	Rough awns	Intermediate-smooth awns	Smooth awns
Observed count.....	850	188	50	2	3	14
Calculated segregation on a basis of two 12:3:1.....	816	204	68	14.25	3.56	1.19

$\chi^2=156.54$   $P$ , very small

The segregation in Table 31 is between a 2-factor difference for roughness of awn and a 3-factor difference for branching of style. The unbranched style group has only 19 plants. However, the unbranched style-smooth awn class is high and the unbranched style-rough-awned class is low when compared with the calculated 12:3:1 ratio. The reverse is true, to a lesser extent, in the branched style group. The above deviation from the calculated 12:3:1 ratio indicates linkage. The dominant factors concerned in this cross enter in the *Coast* parent, and the recessive factors enter the cross together in the *Lion* parent. Therefore, if there is linkage of the factor pairs for branched style and rough awn or unbranched style and smooth awn, it would be in the coupling phase. If such a linkage occurred, the rough-awned plants with branched styles and the smooth-awned plants with unbranched styles would be present in greater numbers than would be expected with independent inheritance. Similarly, the rough-awned plants with unbranched styles and the smooth-awned plants with branched styles would be less numerous than the calculated ratio for independent inheritance. Such a condition is found in Table 31, indicating a possible linkage between the main factor pair for rough awns ( $Rr$ ) and one of the factor pairs for style branching. The data, however, are not sufficient to permit the calculation of the crossover percentage with any degree of accuracy.

#### DISCUSSION

In studying linkage relationships, only those having a direct bearing on the factor pairs considered in this paper will be discussed. A rather extensive list of linkage relationships has recently been made by Daane (2) and Buckley (1).

TABLE 29 —  $F_2$  segregation of long versus short haired rachilla ( $Ss$ ) and intermediate-smooth versus smooth awns ( $R'r'$ )

Item	Number of plants having characters indicated			
	Long-haired rachilla		Short-haired rachilla	
	Intermedi- ate-smooth awns	Smooth awns	Intermedi- ate-smooth awns	Smooth awns
Observed count.....	482	157	161	60
Calculated segregation, 9 3 3 1.....	483 75	161 25	161 25	53 75

 $\chi^2=0.8454$   $P$ , very large

The fit of the observed to the calculated is very good, indicating independent inheritance of the factor pairs  $R'r'$  and  $Ss$ . These data agree with the findings of Sigfusson (12).

Since the factor pair  $R'r'$  for intermediate smooth versus smooth has been found to be inherited independently of the factor pair  $Ss$  for long versus short haired rachilla, the intermediate-smooth and smooth phenotypes may be combined in a study of linkage between the factor pairs  $Rr$  and  $Ss$ .

According to the symbols used in this paper, the Leon parent has the genetic constitution  $rr$ ,  $r'r'$ ,  $SS$ , and the Coast parent has the genetic constitution  $RR$ ,  $R'R'$ ,  $ss$  for the characters roughness of awn and rachilla hairs. The characters went into the cross in the repulsion phase. The dominant factor  $R$  concerned in this study is in the rough class only, while the recessive factor  $r$  is in the intermediate-smooth and smooth classes. The factor pair  $R'r'$  is common to both classes, but should not interfere in the calculations, as it was found to be inherited independently of the factor pair  $Ss$  for long versus short-haired rachilla. The linkage value was calculated on the basis of a (3 1) (3 1) ratio by the use of Immer's tables (8). The observed data are compared with the calculated ratio with a crossover percentage of  $34.63 \pm 1.76$ . (Table 30)

TABLE 30 — Comparison of the observed and the calculated ratios with 34.63 per cent crossing over

Item	Number of plants having character indicated			
	Long-haired rachilla		Short-haired rachilla	
	Rough awns	Intermedi- ate-smooth and smooth awns	Rough awns	Intermedi- ate-smooth and smooth awns
Observed count.....	593	223	259	32
Calculated segregation, 34.63 per cent crossing over.....	576 6	239 4	256 1	34 9

 $\chi^2=1.95$   $P=0.4004$ 

The data in Table 30 indicate a linkage of the factor pairs  $Rr$  for rough versus smooth awn and  $Ss$  for long versus short haired rachilla, with a crossover value of  $34.63 \pm 1.76$ . The above crossover percent-

## GROUP 5 ROUGH VERSUS SMOOTH AWNS

Several workers (12, 7) have reported the linkage of one of the factor pairs for rough versus smooth awn (*Rr*) and long versus short haired rachilla (*Ss*). This linkage is confirmed and the linkage values of all three studies are within three times their probable errors. The cross-over percentage is about 34.

GROUP 6. ALBINO (*A<sub>3</sub>a<sub>3</sub>*) VERSUS GREEN

Only one linkage in this group has been found, that of the factor pair *Xx* for green versus xantha seedlings. The factor pair *A<sub>1</sub>a<sub>1</sub>* is not closely linked with the following factors found in the above-mentioned groups: (1), Non 6-rowed versus 6-rowed, (2), black versus white lemma; (3), hulled versus naked caryopsis; (4), hoods versus awns, and (5), rough versus smooth awn.

In making the studies reported in this paper, *F<sub>3</sub>* data have been used frequently. The possibility of obtaining coupling phases from *F<sub>2</sub>* and *F<sub>3</sub>* segregations when the *F<sub>2</sub>* 3 to 1 ratios can be separated into 1:2:1 ratios by the use of *F<sub>3</sub>* data is discussed in this paper. When one or other of the factor pairs can be segregated into three classes where a single-factor difference determines the character difference, a 1:2:1 ratio may be obtained and by proper grouping of the heterozygous class with one or the other of the homozygous classes, a 3:1 or 1:3 ratio may be obtained and a coupling phase in the *F<sub>2</sub>* used for the determination of linkage. This method cuts down the error, since the small double recessive group obtained in the repulsion phase of linkage is combined with the heterozygous individuals in the coupling phase and the loss of a few individuals of this class has less influence on the linkage determinations when in the coupling phase. An example of this type of classification with the same data is given in Tables 18 and 19, where the interrelationship of 2 row versus non 2 row and green versus chlorina (*Ff*) is studied.

## SUMMARY

In this paper the inheritance of the following character pairs is explained on a simple Mendelian basis. Green versus chlorina seedlings (*Ff*) in Minnesota 84-7, green versus virescent seedlings (*Yy*) in Minnesota 72-8, blue versus nonblue aleurone (*Bl bl*).

A 2-factor difference was found to explain the difference between rough and smooth awns. A 12:3:1 ratio of rough, intermediate-smooth, and smooth-awned plants was found. The symbols *Rr R'r'* were used.

The interrelationship of several botanical characters and chlorophyll deficiencies was studied, with the following results:

(1) The factor pair *Ff* for green versus chlorina seedlings was found to be inherited independently of the factor pairs *A<sub>1</sub>a<sub>1</sub>* for green versus white seedlings in Trebi, *A<sub>2</sub>a<sub>2</sub>* for green versus white seedlings in Colseess, *F<sub>2</sub>f<sub>2</sub>* for green versus chlorina seedlings in Colseess, *Ss* for long versus short haired rachilla, and *Kk* for hoods versus awns.

(2) The factor pair *Xx* for green versus xantha seedlings in Colseess was found to be inherited independently of the factor pairs *Yy* for green versus virescent seedlings in Minnesota 72-8 and *Bl bl* for nonblue versus blue aleurone.

The factor pair *Bl bl* for blue versus nonblue aleurone was found to be inherited independently of the factor pair *Ss* for long-haired

## GROUP 1 NON 6-ROWED VERSUS 6-ROWED

The following plant characters have been described by two or more independent workers as belonging to this group (2). Height of plant, length of awn, early versus late heading, and extension of the outer glume

In crosses between Minnesota 84-7 and Treb1, a linkage has been found between the factor pair  $Vv$ , which distinguishes between the 2-rowed and 6-rowed character, and  $Ff$ , a factor pair for chlorina seedlings first described by Nilsson-Ehle (9) and later obtained from the Minnesota station under the number Minnesota 84-7. This chlorina seedling was found by Nilsson-Ehle to be linked with a white seedling factor pair known as  $A_3a_3$ , albino 3. Hallquist (4) confirmed this linkage and found the linkage of another white seedling factor pair,  $A_4a_4$ , albino 4. He gave the following crossover percentages and arrangement of the genes in the chromosome: Albino 3 and chlorina, 10.2 per cent crossover; albino 4 and chlorina, 3.8 per cent crossover, and albino 3 and albino 4, 12.5 per cent crossover. This would mean that the genes were arranged in the chromosome as follows: Albino 4, chlorina and albino 3. Buckley (1) in a recent paper reported a linkage of the factor pair for 2-rowed versus 6-rowed, with several genes concerned in the development of colored veins on the lemma and one of the two genes concerned with the development of red pericarp. He also lists the chlorophyll-deficient series of Nilsson-Ehle and Hallquist as possibly forming a fifth linkage group. The data presented in this paper place this group in the linkage group with 6-rowed versus 2-rowed. However, the arrangement of the factors in the chromosome has not yet been determined. The factor pair for the row character may be either to the right or to the left of the factor pair for chlorina.

## GROUP 2 BLACK VERSUS WHITE LEMMA AND PERICARP

Several factor pairs are located in this group, but none so far has been found independently by two workers. However, it has been clearly shown by Robertson (10), Sigfusson (12), and Buckley (1) that the factor pair  $Ss$ , for long versus short-haired rachilla is inherited independently of the factor pair  $Bb$  for black versus white pericarp.

## GROUP 3 HULLED VERSUS NAKED CARYOPSIS

Only one factor pair has been reported by two workers in this group that is dense versus lax head (2).

## GROUP 4 HOODS VERSUS AWNS

Buckley found a linkage between the factor pairs for hoods versus awns and  $Bl bl$  for blue versus nonblue aleurone. This linkage is confirmed in the present paper. However, the linkage value here reported is smaller than that found by Buckley (1). It was also shown that the chlorophyll-deficiency factor pairs  $A_c a_c$  and  $X_c x_c$  which are closely linked and which were thought to be loosely linked to the factor pair  $Kk$  for hoods versus awns, are evidently not linked to the factor pair  $Bl bl$  for blue versus nonblue aleurone. This is easily understood when it is remembered that the crossover value reported by Robertson (10) from a segregation of three classes was 45.09 per cent. If  $Kk$  was also linked with  $A_c a_c$ , a linkage of  $A_c a_c$  and  $Bl bl$  should have been obtained with a crossover value of about 25 per cent.

# A PHOTOGRAPHIC LIGHT BOX FOR USE IN AGRICULTURAL RESEARCH<sup>1</sup>

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## INTRODUCTION

The difficulty experienced in attempts to obtain a constant and always uniform source of light for photographing diseased fruits and similar specimens led the writer to devise a more satisfactory method of illumination. The result of considerable experimenting was the production of a light box which has proved to be most satisfactory for its purpose, and its use has resulted in the saving of considerable time and material, as well as in obtaining better results than had been possible before. This light box has also proved to be useful in lantern-slide production, natural-color photography, and in low-power photomicrographic work where upper-field illumination is desired.

## APPARATUS

The illuminating device as designed for use with a Leitz vertical camera (pl. 1, A) consists essentially of a box, square at the top and rectangular at the base, with the lower portion of two sides extended 3 inches at one end to make this part of the box project beyond the square upper part. The box is provided with a removable toppiece carrying a shielded aperture for the camera lens. The upper section of the box carries four 50-watt light bulbs which serve as a source of upper illumination, while two bulbs of equal size at the bottom of the box provide illumination from beneath the subject. The projecting right end of the box is hinged to provide ready access to the interior of the apparatus so that the specimen-supporting fixtures may be manipulated and the specimens arranged during the focusing process. Four grooves are cut into the inside faces of each of the two large sides of the box, and the specimen-supporting fixtures are carried in these grooves. These four grooves make it possible to adjust the distance of the subject from the lens and thus obtain a suitable magnification without moving the lens more than a slight amount, if at all. This is important, as the field becomes restricted if the lens is moved any considerable distance upward. The lights are controlled by three switches, one line switch in the cord a short distance from the box and two tumbler switches attached to the outside of the box. The upper switch controls the four bulbs on the upper circuit, and the lower switch controls the lower two bulbs. The line switch is used to control the lights while a plate is being exposed, the other two being manipulated only when the line switch is off. This precaution is necessary to prevent any vibration of the subject while it

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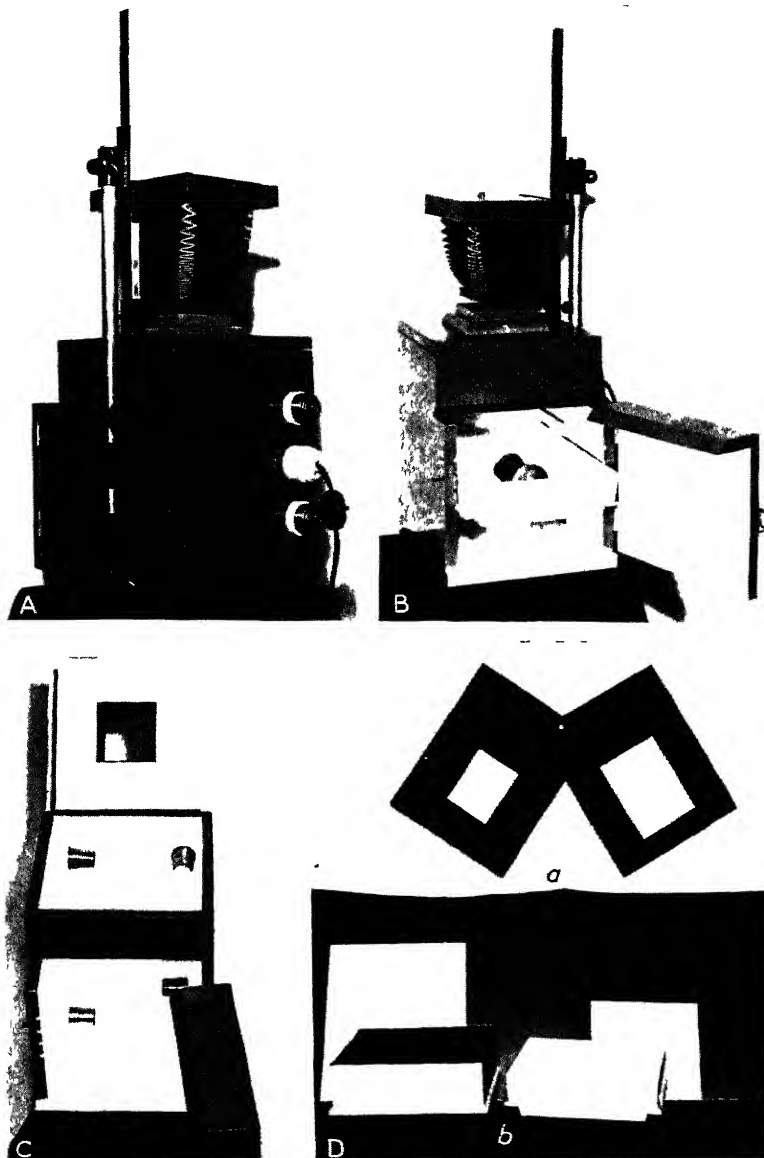
The factor pair *Bb* for black versus white glumes was inherited independently of the factor pairs *Rr R'r'* for rough versus smooth awn and *Gg G'g' G''g''* for branched versus unbranched style. The factor pair *Ss* for long versus short haired rachilla seemed to be inherited independently of the factors for branched and unbranched style. This was explained on the hypothesis that the factors may be located at the extreme ends of the chromosome.

Linkage was found between the following factor pairs

- (1) *Ff* for green versus chlorina seedlings and *Vv* for non 6-rowed versus 6-rowed. A crossover value of  $18.3 \pm 0.74$  per cent was found.
- (2) *Ff\_c* for green versus chlorina seedlings in Colless and *Y\_y\_c* for green versus virescent seedlings in Coast. A crossover value of 29.3 per cent was found.
- (3) *Bl bl* for blue versus nonblue aleurone and *Kk* for hoods versus awns. The crossover value was  $22.58 \pm 0.82$  per cent.
- (4) *Ss* for long-haired versus short-haired rachilla and *Rr* the main factor pair for roughness of awn. The crossover percentage was 34.63.
- (5) There was also an indication of possible linkage between the rough-awn factor pair and some of the factors for branched and unbranched style.

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Photographic light box and accessories. A, Photographic light box in position, showing manner of fitting vertical camera support and the switch arrangement, B, interior view of box showing the manner of placing specimens for photographing, C, interior view of light box, showing the arrangement of light bulbs, D, accessories for use with light box, the masks for making lantern slides are shown in *a*, while the velvet-lined box, the reflector tray, and the pieces of flashed opal and frosted glass appear in *b*

is being photographed. Three pieces of accessory apparatus (pl. 1, D) are used to support the objects to be photographed; two of these are used when a photograph with either a white or gray background is desired, and the third is used only when a completely black background is wanted. The first of these is a 4-sided reflector apparatus (b) which carries a sheet of flashed opal glass at its base and a second sheet of frosted glass over this. The flashed opal glass serves to give satisfactory diffusion of the light from below, but because of its smooth surface it must be covered with a sheet of frosted glass to eliminate objectionable glare from the lights. The second equipment is used when larger objects are to be photographed, or when lantern slides are to be made. This equipment consists of two large sheets of flashed opal and frosted glass (b) cut to fit directly into the grooves in the sides of the box. It is not altogether necessary to use the tray at any time, as these two large sheets of glass may be employed for the same purposes for which the tray is used. The greater convenience of the tray, together with some advantage in illuminating the sides of deep specimens, seems, however, to make its use desirable. A deep, black-velvet-lined box which fits directly into the side grooves serves as a support when a black background is desired.

#### METHOD OF OPERATION

All of the more common types of photographic plates have been used with the apparatus, but the ones most generally satisfactory have been those with panchromatic emulsions. Orthochromatic plates also give good results, but require several times the exposure necessary for panchromatic plates. The process panchromatic plate is also quite useful where it is desired to accentuate the contrast. Filters have not been used as much as with daylight, but it has been found that the Wratten K 2 and K 3 filters give no appreciable correction. Difficulty was experienced in obtaining the desired contrasts with such subjects as apple and peach leaves and fruits showing spray-injured or diseased regions in which the necrotic or chlorotic areas were light brown, red, or yellow. The use of the proper filter as determined by observation of the object through a filter test chart resulted in securing satisfactory photographs. The Wratten A, B, and G filters are used where it is desired to obtain clear definition with such objects having slight contrasts between greens, reds, brown, and yellow.

The 100-mm lens is used for practically all the work, as it will cover a 5 by 7 inch plate at the distance it must work from the objects in the box. This lens gives natural-size reproduction as well as a certain degree of enlargement or reduction. The shorter focal-length lenses will not cover as large a field in the necessary working range.

#### PHOTOGRAPHING WITH A WHITE BACKGROUND

The white background, as used with the apple target-spot material illustrated (pl. 2, B), is the most generally satisfactory and the most commonly used. It may be employed with any opaque or nearly opaque object, such as fruits, twigs, leaves, tubers, roots, and similar specimens, unless they are very light colored. The object to be photographed is placed directly on the frosted glass (pl. 1, B), which may

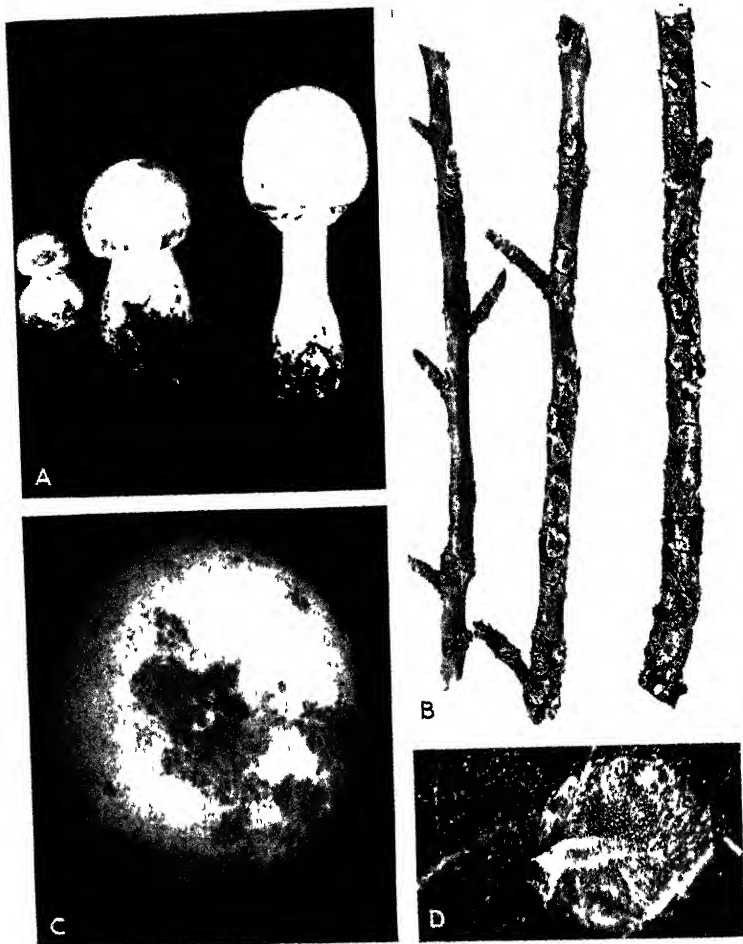
be either the one used in the reflector tray or the large glass. The flashed opal glass must be placed beneath the frosted glass to obtain even illumination from below. The upper, or both the upper and lower, lights are switched on and the arranging of the specimens and the focusing of the camera completed. A diaphragm aperture of approximately  $f\ 48$  is usually used for the exposure. The lights are switched off, the plate holder inserted, and the protecting slide withdrawn. The lower switch is set at "on," the upper one at "off," and the line switch turned on for approximately two seconds, provided a panchromatic plate without a filter is used. The line switch is then turned off, the upper switch also set at "on," and a second exposure with both sets of lights for approximately one second is given. This completes the exposure. Opaque objects, or those nearly so, may be silhouetted with the lower lights and a small lens diaphragm, insuring a satisfactory white background as well as destroying any background shadows. With many objects it is not necessary to use the additional background exposure. In such cases both sets of lights are used together for the single exposure. The periods of exposure vary but little, and consequently after they have once been determined for a certain plate and developer, a high percentage of satisfactory plates may be expected.

#### PHOTOGRAPHING WITH A GRAY BACKGROUND

Light to medium gray backgrounds may be produced by inserting a sheet of transparent red paper between the frosted and flashed opal glasses and proceeding in about the same manner as for a white background. A medium-gray background may be obtained with the use of the upper set of lights only, the use of the lower set of lights for varying periods will produce lighter-gray backgrounds. The red paper used in wrapping film packs and other photographic materials is quite satisfactory. The dark-gray background, as used in photographing the apple injured by summer-oil spraying (pl. 2, C), was satisfactorily produced by inserting a sheet of black paper between the two sheets of glass and using only the upper set of lights. The rough surface of the glass will reflect sufficient light to give a gray background, and at the same time no photographic impression of the paper will be obtained as would be the case if the subject were placed directly upon the paper. The gray background is most useful with objects which contain considerable contrast, making either the white or black backgrounds somewhat unsatisfactory.

#### PHOTOGRAPHING WITH A BLACK BACKGROUND

Completely black backgrounds are desirable only when sharp contrast is desired, as was the case with the mushroom, *Lepiota naucina* (Pl 2, A). Such a background is obtained by the use of the box lined with black velvet. The subject to be photographed may be placed directly upon the bottom of the box, or upon some small support which is covered by the object itself. Care must be taken to remove all lint and light-colored particles from the surface of the velvet. The upper set of lights are used alone and the usual exposure given. If these precautions are observed an even black background, free from evidences of the support, will be obtained.



Photographs made with the aid of the light box, illustrating the different types of background obtainable, and also showing the possibilities of the light box in photomicrography. A, *Lepiota naucina* on black background, B, target canker on apple twigs, white background, C, spray injury on apple, gray background, D, young larva of *Cydia pomonella*. D is X 25.

the connecting groove in the left end, are designed to hold the wiring system, which is completely covered in the finished box. These grooves fit together to make continuous channels when the pieces are assembled. The four  $\frac{1}{4}$ -inch grooves in each of the two sidepieces are for holding the accessory fittings, as has been previously mentioned.

The back, front, and left sidepieces are nailed to the bottom through the sides, as the bottom fits inside the box and even with the lower

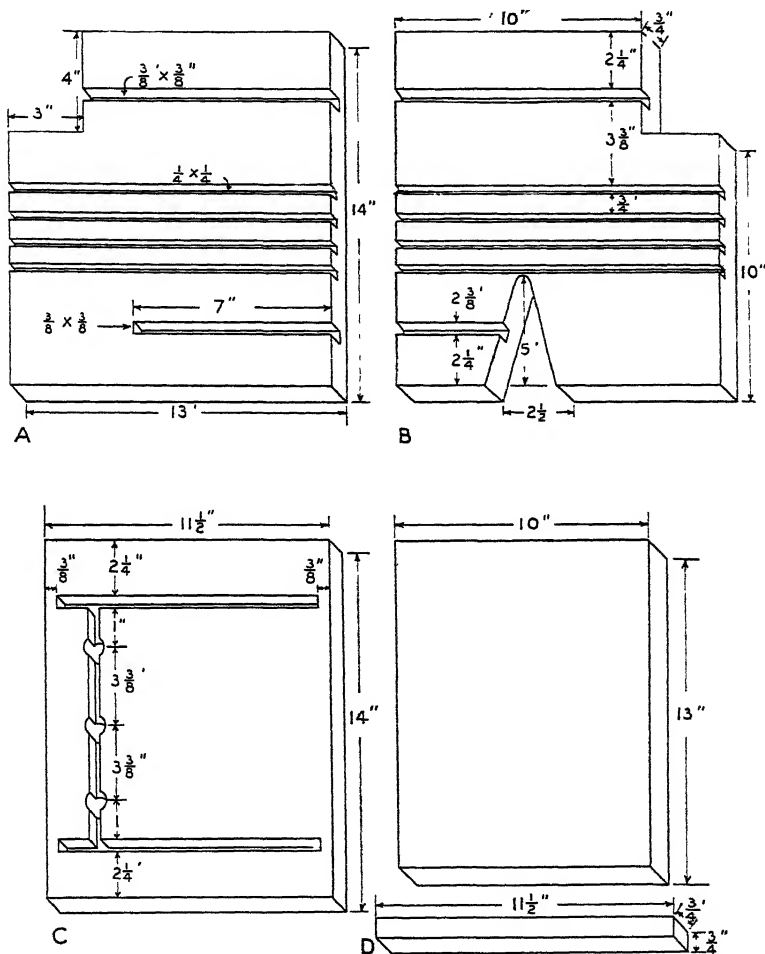


FIGURE 1—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box A, Front, B, back, C, left side, D, bottom

edges of the side and end pieces. The upper piece of the right-end assembly is nailed across the upper inset of the two sidepieces. The other two right sidepieces are nailed together as illustrated in Figure 2, B, to form the door of the box. This door is hinged to the side away from the operator, or to the sidepiece designed to fit up against the upright support of the camera. It should be mentioned here that it will be necessary to cut a groove in the outside face of the sidepiece

## NATURAL-COLOR PHOTOGRAPHY

The Autochrome or Agfa color plates may be used with the light box for taking photographs in natural colors. The writer has not found it necessary to use a filter, the light from the vacuum bulbs producing very satisfactory results without correction. Nitrogen-filled tungsten bulbs would no doubt be desirable if a great deal of this kind of work were to be done. The period of exposure ranges from 20 seconds to over a minute, depending upon the density of plate desired.

## LANTERN-SLIDE PRODUCTION

The light box may be used in making lantern slides from plates not larger than 5 by 7 inches in size. For this purpose a sheet of black cover-stock paper is cut to the size of the large pieces of glass and a correctly centered section slightly smaller than the plate with which it is to be used is cut from the sheet (Pl 1, D, *a*). The section removed must be centered beneath the camera lens. This paper mask is placed between the two large sheets of glass, the three are then slid into two of the parallel grooves which run horizontally between the upper and lower sets of light bulbs (pl 1, B and C), and the negative placed over the aperture in the paper. The lower lights are switched on and focusing completed, after which the exposure is made in the usual manner.

## PHOTOMICROGRAPHIC ILLUMINATION

The light box may be used quite successfully for upper-field illumination in low-power photomicrographic work (pl 2, D) when other and more convenient methods are not available. To use the box for this purpose, the lower bulbs are removed and the microscope is placed on the bottom of the box and centered. The tube should be extended to the proper length and the top of the box replaced. The microscope may be too low, in which case it may be raised by placing as many sheets of cardboard beneath it as are necessary to raise it to the desired height. The camera is then fitted to the microscope as usual, the upper lights turned on, and the microscope focused. It is necessary under these circumstances to manipulate the microscope controls through the open door of the box. An exposure of approximately 30 seconds is required at a magnification of 25 diameters. The light box illuminates the field evenly and produces no shadows, shading of the field being necessary if some shadows are desired with light objects showing little contrast.

## CONSTRUCTION AND FITTING OF THE BOX

The light box is not difficult to construct, and may be made by any woodworking or cabinet shop. The total cost, including labor and materials, should not exceed \$20 or \$25.

Three-quarter-inch white-pine lumber is used in the construction of the box. This makes a very substantial piece of equipment and allows sufficient thickness to cut the wiring and slide grooves. Detailed drawings of all the parts of the box are given in Figures 1-3. The drawings and measurements are based on butt-end construction, although mitered construction may be used if preferred. The  $\frac{3}{8}$ -inch grooves at the top and bottom of the individual pieces, together with

and 1 inch above the upper surface of the toppiece. Three-quarter-inch quarter-round molding is fitted around the top of the tube to brace it and give the top a finished appearance. The lower side of the toppiece is fitted with a flange made of triangular stock  $1\frac{1}{2}$  inches on each of two sides (Pl 1, C, and fig 3, B). This piece holds the top

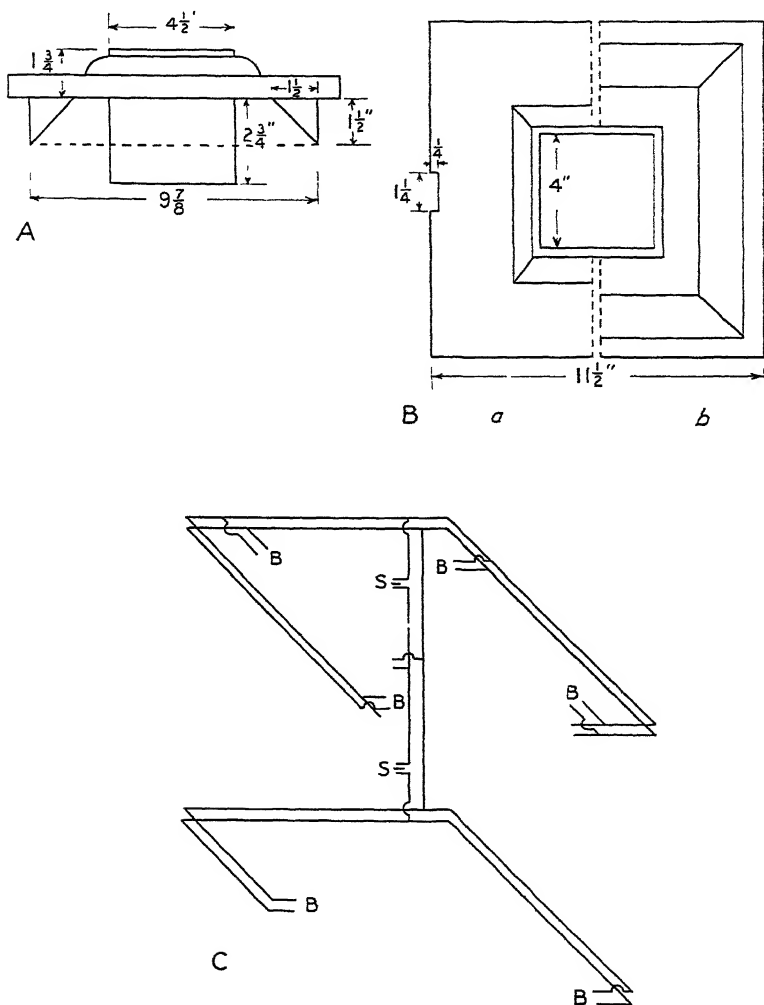


FIGURE 3—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box, and the arrangement of the light bulbs. A, Cross-sectional view of the top, B, toppiece with upper side shown at *a* and lower side at *b*, C, diagram of wiring in which B stands for bulb and S for switch

in position and also serves as a reflector above and back of the bulbs. The toppiece should not fit too tightly or it may bind after painting.

The box is wired with double-strand insulated wire, which is placed around in the wiring grooves in the box and secured with insulated staples. A loop of wire should be left about 1 inch from each corner

fitting against the camera support in order to center the camera properly. This groove extends upward from the apex of the triangular cut in the bottom of the sidepiece, but this groove has not been illustrated as the fitting will have to be made to the individual camera (Pl 1, A). The small  $\frac{3}{4}$  by  $\frac{3}{4}$  by  $11\frac{1}{2}$  inch piece is nailed across the right end to the bottom piece and extends the bottom out even with the outer edge of the door. When assembled, the inside dimensions

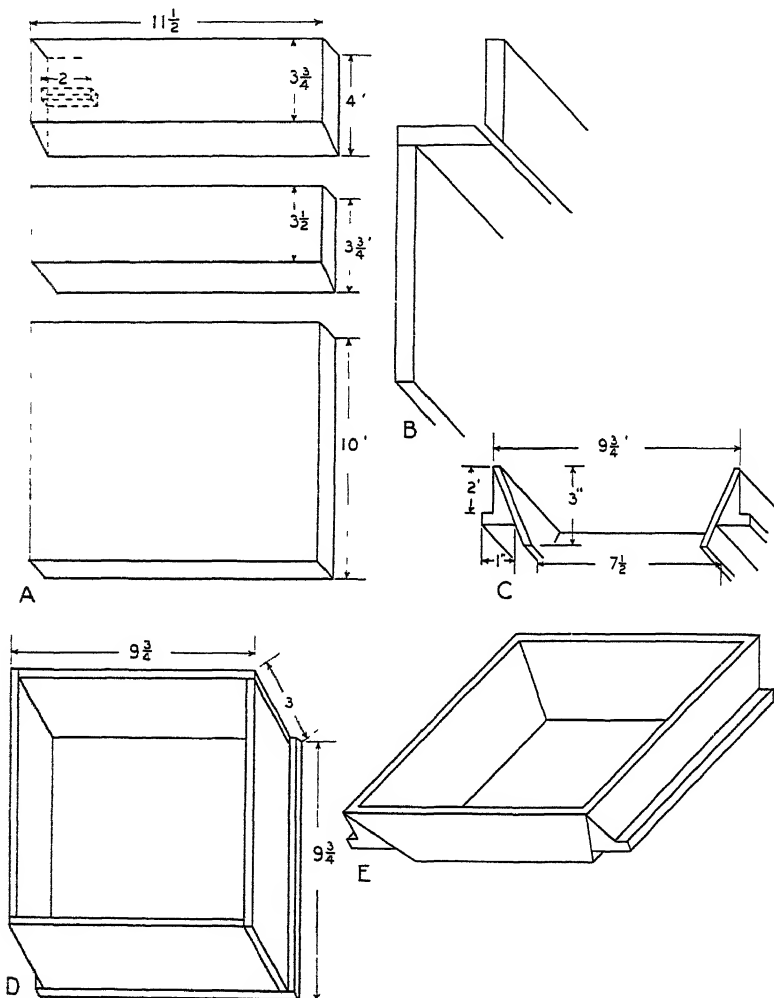


FIGURE 2—Detailed drawings showing the dimensions and construction of certain parts of the photographic light box. A, Right side. B, detail of right-side construction. C, cross section of the reflector. D, black-background box. E, reflector tray.

of the box are 10 by 10 inches at the top and 10 by 13 inches at the bottom. The outside dimensions are  $11\frac{1}{2}$  by  $14\frac{1}{2}$  inches. The top-piece is  $11\frac{1}{2}$  inches square, having a section  $4\frac{1}{2}$  by  $4\frac{1}{2}$  inches cut from the center. A square tube constructed of  $\frac{1}{4}$ -inch plywood is fitted in the hole in the top so that it projects  $2\frac{3}{4}$  inches below the lower surface

night, and so long as other factors are kept the same the period of exposure for best results will not vary appreciably. This results in a great saving in time and photographic supplies.

In addition to being of use in ordinary indoor photography, the box may be used for making lantern slides and in low-power photomicrographic work. The construction of the box, together with its method of operation, is described in detail.

and at the ends of the two lower grooves for later attachment to the light sockets. A single loop is pulled through the upper and lower holes in the left end piece for attachment to the two circuit switches. A double loop is pulled through the center hole to be attached to the rosette to which the lead-in wire is attached. After the wiring has been completed, but before any fixtures have been attached, the wiring grooves are filled with plastic wood material and this allowed to dry thoroughly and harden. The small irregularities remaining are then smoothed over with a thin paste of plaster of Paris and after this has dried and again been smoothed down, the box is ready to be painted. After painting, open-bottom receptacles are fitted at the bulb locations and the wiring brought up through them and attached to the keyless sockets. This assembly of bottom-wired receptacles and keyless sockets places the filaments of the bulbs at approximately the centers of the sides. The switches, rosette, and hinges are also attached after painting has been completed. The arrangement of the bulbs and switches is shown in Plate 1, A and C. The wiring diagram is given in Figure 3, C.

The interior of the box is painted white, as is the reflector tray. Lacquer has proved to be better than enamel for this purpose. The interior of the square lens-receiving tube or aperture is lined with black velvet, although dull black paint should be satisfactory.

The reflector tray is made of  $\frac{1}{4}$ -inch plywood, as is the black background box. The reflector tray is constructed with sloping sides, the dimensions at the top being  $9\frac{3}{4}$  by  $9\frac{3}{4}$  inches and  $7\frac{1}{2}$  by  $7\frac{1}{2}$  inches at the bottom. The tray has no other bottom than the two pieces of glass which serve as a transparent base. The two pieces of glass are cut 8 inches square, thus fitting near the bottom of the tray. The tray is fitted with projecting tongues on two sides (fig 2, C and E) to fit into the side grooves. The black background box is a plain open-top box  $9\frac{3}{4}$  inches square and 3 inches deep (fig 2, D) with the bottom edge projecting a short distance on each of two sides in order to engage in the grooves in the box. The interior of the box is lined with a good grade of black velvet. This box may also be used quite satisfactorily outside the box. The two large pieces of frosted and flashed opal glass are each cut  $10\frac{5}{8}$  by 13 inches in size.

The light box as described is designed to operate with the Leitz vertical camera, although the design may be readily adapted to other cameras. The principal precautions to observe are to see that the over-all height of the box is not greater than the height to which the lower end of the camera rail can be raised, and to determine the largest size box that can be centered beneath the camera.

#### SUMMARY

The light box described in this paper has proved very satisfactory as a source of illumination for photographing diseased fruits and similar specimens. It provides a simple and inexpensive means by which shadows, high lights, and cross lights can be eliminated and at the same time enables the operator to secure the color of background that is best suited for the object to be photographed.

Since the illumination comes entirely from artificial sources, daylight being excluded, the light is always uniform. This makes it possible for one to do photographic work at any time of day or at

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## THE INHERITANCE OF THE WHITE BURLEY CHARACTER IN TOBACCO<sup>1</sup>

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### INTRODUCTION

Progress in the development of improved strains of tobacco is limited by a lack of exact knowledge relative to the inheritance of specific characters which make up the varietal complex. In the case of the White Burley variety, the basic character is an apparent reduction in chlorophyll, which renders it economically important. Any attempt, therefore, at the improvement of this variety by hybridization must take into account the inheritance of this character. The present study was, in part, prompted by this consideration; for, although White Burley tobacco has been the subject of some genetic investigation in the past, the inheritance of the "white" character has remained obscure.

White Burley tobacco originated in 1865, presumably as a mutation from the green-colored variety Little Burley (Mathewson (10)).<sup>3</sup> In appearance, White Burley seedlings are characteristically somewhat lighter green in color than seedlings of green varieties. The stems of White Burley seedlings in particular are clear white and have a glossy appearance in contrast to the dull greenish-white stems of green varieties.

For a few weeks after the seedlings are transplanted, under good growing conditions the color difference between White Burley and green<sup>4</sup> varieties becomes less distinct, especially while the plants are growing rapidly. With the approach of maturity, however, White Burley tobacco loses much of its green color, particularly in the lower leaves and in the stem. If the usual commercial practice is followed, and the plants are topped by breaking off the stem several nodes below the seed head, the loss of chlorophyll in white Burley plants is increased, and within a short time the plants become light yellow in color. Except for a slight mottling of the leaves as they ripen, green varieties retain their color under this treatment.

### REVIEW OF LITERATURE

A survey of the literature reveals that in almost every plant genus which has been subjected to intensive genetical investigation, heritable chlorophyll deficiencies have been reported, and in many cases a

<sup>1</sup> Received for publication May 8, 1931, issued May, 1932. Paper No. 120 from the Departments of Genetics and Horticulture, Agricultural Experiment Station, University of Wisconsin.

<sup>2</sup> The author desires to express his sincere appreciation to Dr. R. A. Brink and to Dr. James Johnson for many helpful suggestions in the course of the work. The writer is also particularly indebted to Doctor Johnson for his generous provision of materials and for the unpublished data appearing in this manuscript.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 493.

<sup>4</sup> The term "green" as used in this paper refers to the normal genetic green color of leaves and stalks, and "white" refers to the white or yellow color characteristic of White Burley tobacco.



## MATERIALS AND METHODS

Dr James Johnson laid the foundation for the present investigation in 1916 while engaged in tobacco investigations at the Wisconsin Agricultural Experiment Station. In the course of studies on the inheritance of disease resistance in tobacco he (4) crossed a number of green lines with pure lines of White Burley. In subsequent generations from these crosses segregation for color of plant was observed and recorded so that by 1928, when the writer became interested in this material, a substantial body of data dealing with the inheritance of the chlorophyll deficiency had been accumulated.

## VARIETIES USED

The majority of Johnson's observations were made on progenies of the cross Little Dutch  $\times$  White Burley, strain Judy's Pride. Since seed of the  $F_1$ ,  $F_2$ , and  $F_3$  generations from this cross were available as well as seed of the  $F_1$  backcrossed to White Burley, it seemed advisable to use this material in the further analysis of the White Burley character. Little Dutch, the green parent in this cross, is an Ohio strain grown for cigar-filler tobacco in that State.

Judy's Pride is a typical strain of stand-up White Burley tobacco such as is grown extensively in Kentucky. Under optimum conditions it makes a very vigorous growth.

Other green and white crosses made during the course of the investigation involved the following pure-line varieties: SB9AAX, a stand-up White Burley strain resistant to black root rot, Havana 142, a green line of cigar-binder tobacco extensively grown in Wisconsin, Xanthia, a green Turkish strain with small oval leaves, 180A31, a green strain of cigar-binder tobacco; and White Burley, strain Judy's Pride.

## FIELD METHODS

In general, the procedure in these earlier investigations was that common to commercial tobacco growing in Wisconsin. Seed was sown in steam-sterilized seed beds early in April, and the seedlings transplanted to the field in June. No conscious selection was exerted in the choosing of plants from the seed bed except as in commercial practice—that is, the earliest plants, and therefore usually the largest plants of an even size, were taken. Counts on segregating progenies were made after topping, when the phenotypic difference between genetically green and genetically white individuals was greatest. In some cases the plants left in the seed beds were permitted to grow until they could be classified; in certain other cases, after field planting the majority of plants were removed from the seed bed and discarded, permitting those remaining to grow until their character could be determined.

## METHODS USED IN GREENHOUSE TESTS

With the field method the size of populations was limited by available land and by facilities for culture; consequently other methods for the study of the white character in larger numbers were sought.

Early in 1929 a method was devised for the identification of the chlorophyll deficiency in the seedling stage in the greenhouse. It was found that pure-line green and pure-line White Burley plants gave a different reaction to a period of total darkness at high temperature.

satisfactory genetic analysis has been made. In the genus *Nicotiana* four cases may be cited

Lodewijks (8) reported the occurrence and behavior of certain "aurea" forms of *Nicotiana tabacum* which originated as mutations in his experimental fields at Klaten, Java, in 1908 and 1909. Two self-pollinated aurea plants gave rise to two aurea groups, similar in appearance but apparently distinct in genetic behavior. Several self-pollinated generations of group 1 aureas yielded 75 per cent aurea and 25 per cent green. Aureas of group 2, however, yielded 35 per cent aurea and 65 per cent green. Reciprocal crosses between the aureas and the green types made by Lodewijks gave the following results: In group 1, aurea  $\times$  green yielded 83 per cent aurea in  $F_1$ , and the reciprocal cross, green  $\times$  aurea, 48 per cent aurea. For group 2, crosses with aurea as the female parent gave in  $F_1$  48 per cent aurea, and green  $\times$  aurea gave 43 per cent aurea.

Since he was unable to establish true breeding aureas of either group, Lodewijks concluded that his aureas, like certain of Baur's Antirrhinum (2), existed only in the hybrid form.

Lubimenko and Palamartchouck (9) studied the different amounts of chlorophyll present in certain Russian and American varieties of *Nicotiana tabacum* as determined by chemical analysis. Data for the parents and the  $F_1$  of a series of crosses were reported, but no attempt was made at factorial analysis in  $F_2$ .

Allard (1) studied an aurea form of *Nicotiana rustica*. In crosses with the green form of this species he found that the aurea form behaved as a simple Mendelian recessive.

Kajanus (5), working in Sweden, reported the results of crossing White Burley tobacco obtained from Virginia, with a green variety native to the Netherlands. The  $F_1$  of this cross was green, like the green parent, and in  $F_2$  the following distribution was obtained:

	Green	White
Observed.....	5, 037	229
Expected (15 : 1).....	4, 937	329 $d=100 \pm 11$ 85 <sup>5</sup>

In the  $F_3$  generation, although numerical ratios are not given, constant green, constant white, and segregating families were obtained with the truebreeding green progenies constituting the majority.

In the first three cases cited, the chlorophyll-deficient characters under consideration can not be regarded as being identical with the White Burley character, although they are apparently similar in appearance. The aurea character of Lodewijks is, in fact, quite different since it behaved as a dominant and upon self-pollination gave rise to progenies segregating for aurea and green.

The aurea character reported by Allard (1, p 234) in *Nicotiana rustica* is essentially similar to White Burley in appearance but is a monohybrid and in a different species. The crosses of Lubimenko and Palamartchouck were not carried through the second generation and consequently do not show the number of genetic factors involved.

Only the paper of Kajanus deals with the White Burley character. Since his White Burley variety was obtained from Virginia, it may be safely considered as belonging to the same chlorophyll-deficient group as the varieties used in the present investigation. The evidence of a simple dihybrid relationship between the green and white varieties used by Kajanus is, however, not conclusive.

<sup>5</sup> Apprehension of probable error made by the writer from Kajanus' data.

## INTERPRETATION OF EXPERIMENTAL RESULTS

In the interpretation of experimental results, probable errors and the  $\chi^2$  test for goodness of fit as outlined by Kirk and Immer (6) were employed.

Probable errors were taken from tables based on standard formulae, and values of  $\chi^2$  were taken from tables by Fisher (3).

FIELD RESULTS WITH LITTLE DUTCH  $\times$  WHITE BURLEY

The data presented in Table 1 were brought together from the field notebooks of Doctor Johnson late in 1928. A small field planting of certain segregating progenies from cross 63 had been made by the writer during the summer of the same year. The results of this planting and the field results of the  $F_2$  population planted in 1929 are included in these totals for the sake of completeness.<sup>6</sup>

Table 1 presents a summary of field segregation in  $F_2$  and  $F_3$  of Little Dutch  $\times$  White Burley and in the back cross of the green  $F_1$  to recessive White Burley. Inspection of the data listed reveals a rather poor fit on the basis of dihybrid segregation. There is in every case a deficiency of recessives considerably greater than ordinarily expected. Furthermore among the 20 progenies that make up the totals in Table 1, all but 3 were in turn recessive-deficient. In addition to the segregating  $F_3$  families a number of true-breeding green and true-breeding white  $F_3$  progenies were recovered by Johnson.

TABLE 1.—*Field counts on Little Dutch  $\times$  White Burley (cross 63), 1928*

Designation	Progenies	Plants		Ratio	Deviation	Dev P E	
		Green	White				
	Number	Number	Number	Per cent			
63F <sub>2</sub>	4	4,371	187	3.7	15.1	-116.6±11.0	10.6
63F <sub>3</sub>	8	1,732	92	5.0	15.1	-22.0±7.0	3.1
63F <sub>3</sub>	2	377	79	17.3	3.1	-35.0±6.2	5.6
63F <sub>1</sub> ×WB	6	783	195	19.9	3.1	-49.9±9.1	5.4

In general, two interpretations may be made of data of this character. (1) Inheritance may be of a simple Mendelian nature, and environmental factors may be considered as responsible for the elimination of recessives at some early stage, or (2) inheritance may be more complex, involving modifying factors and possibly linkage.

Attempts were made to check the recessive-deficient ratios observed by classifying the plants remaining in the seed beds at the conclusion of field planting to determine if selection based on a differential growth rate of green and white seedlings could be responsible for the deficiencies. In cases in which sufficient plants remained to yield a significant tally, ratios closely approximating those observed in field planting were obtained.

Examination of  $F_2$  results and the data from the back-crossed  $F_1$  generation disclosed no evidence of difference in reciprocal crosses, which rules out any hypothesis assuming differential pollen-tube growth.

<sup>6</sup> Typed copies of the data from which summary tables appearing in this manuscript were taken are available for inspection.

The chlorophyll-deficient character of White Burley seedlings was markedly accentuated by the treatment, whereas pure-line green seedlings were only slightly affected. Experiments with both pure-line and segregating material led to the adoption of a standard etiolation period of seven days at a temperature of 90° F for seedlings approximately 4 inches in height.

The effectiveness of the etiolation period was governed, apparently, by two factors—temperature and the physiological state of the plants as evidenced by their rate of growth. At temperatures of 50° to 60° F, 14 or 15 days of total darkness were required to etiolate White Burley seedlings completely, as compared with 7 days at 90°. Furthermore, plants not in a state of rapid growth were much less uniform



FIGURE 1—A typical flat of seedlings after etiolation, progeny 63-22♂XV B ♀ 65 green, 55 white

in their reaction to the etiolation. For this reason, during the darker winter months of December and January, artificial light was used to prolong the daily growing period.

The accuracy of this method was thoroughly checked by experiment with pure green and pure white lines in the greenhouse. In the summer of 1929 a quantity of material that had been classified by the etiolation method was transplanted to the field. Reclassification of this material under field conditions showed that no errors had been made.

Analysis of progenies from various crosses was begun in November, 1929. Seed was sown broadcast in stock flats on soil steam-sterilized for 30 minutes at 98° to 100° C, and seedlings were transplanted to unsterilized composted soil when about 1 inch in height, and grown to sufficient size for etiolation. Figure 1 shows a typical flat of seedlings of a back-crossed progeny after etiolation.

The agreement between observed and expected results in the distribution is excellent. This progeny test, involving the green segregates only, provides strong evidence of the dihybrid nature of the material. The  $F_3$  tests are summarized in Table 4. Data for the greenhouse counts on the 53 nonsegregating  $F_3$  families totaling over 24,600 plants are not included.

TABLE 4—*Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63), the  $F_3$  progenies segregating for one and for two factors*

Designation	Progenies	Plants			Ratio	Deviation	Dev P E
		Green	White				
	Number	Number	Number	Per cent			
63F <sub>3</sub>	31	19,677	1,105	5.3	15.1	-193.9 ± 23.5	8.3
	23	13,139	3,476	20.9	3.1	-677.8 ± 37.6	18.0

Examination of the  $F_3$  families segregating for two factors revealed that 26 of the total 31 progenies were recessive deficient. Among these individual progenies the extent of the recessive deficiencies was not great since only two progenies had deviations as large as three times their respective probable errors. Furthermore, in but two of the five families showing an excess of white segregates were the numbers sufficiently large to merit individual consideration. For the behavior of these two, 63-57 and 63-78, no good explanation can be advanced. It was noticed, however, in comparing the  $F_3$  families with the results of back crosses of the parental  $F_2$  that the back cross of the green  $F_2$  plants in both of these cases gave populations deficient in recessives.

Additional evidence of the cumulative nature of the recessive deficiencies was supplied by the  $F_3$  families segregating approximately 3 green : 1 white, also summarized in Table 4. Twenty-one of the 23 families tested were recessive deficient, and all progenies involving more than 600 plants showed deficiencies greater than three times their respective probable errors. On the basis of probable errors one might conclude that larger deficiencies occurred in the progenies segregating for a single factor than in those segregating for two factors. Actually, however, the percentage elimination of recessives based on the total number expected in the two cases is nearly the same—16.3 per cent recessive deficiency for the former, and 15.7 per cent for the latter.

In the back crosses of the parental  $F_2$  selections summarized in Table 5 the percentage of recessive elimination was considerably less, namely, 7.1 per cent for progenies segregating 3 green : 1 white, and 2.2 per cent for those segregating in a 1:1 ratio. Although the inference to be drawn from this behavior is not wholly clear, it appears that the degree of recessive deficiency is influenced by the source of the material.

GREENHOUSE TESTS OF  $F_2$  POPULATIONS

With the development of the greenhouse technic for the identification of the White Burley character in the seedling stage, the 1929 growing season was devoted to the production of suitable progenies for greenhouse analysis. A large planting of  $63ZZZF_2$  was made from which some 250 green plants were selected at random and self-pollinated. Over 100 of these selections were also back crossed to pure-line White Burley to provide an additional check on their genetic constitution.

In addition  $F_1$  plants from the cross  $63ZZZ$  were self-pollinated and back crossed to White Burley.

In Table 2 are summarized the results of the greenhouse analysis of 12  $F_2$  families, 10 of which were obtained from self-pollinated plants of  $63ZZZF_1$ . Of the 12 populations investigated, 10 were recessive deficient on the basis of 15:1 segregation, and in 4 of these cases the extent of the deviations was well beyond the limits of variability expected in random sampling. In the total of these populations involving more than 18,000 plants, the cumulative nature of recessive deficiencies in individual populations is apparent.

TABLE 2—Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63) and other green  $\times$  white crosses, segregation in the  $F_2$  generation

Designation	Progenies	Plants				Deviation from 15:1 ratio	Dev P E
		Green		White			
		Number	Number	Number	Per cent		
63F <sub>2</sub>	12	17,543	948	5.1	-210 $8 \pm 22$	2	9.5
224F <sub>2</sub>	2	1,397	78	5.3	-14 $2 \pm 6$	3	2.3
232F <sub>2</sub>	4	1,680	85	4.8	-25 $3 \pm 6$	8	3.7
233F <sub>2</sub>	2	921	50	5.1	-10 $7 \pm 5$	1	2.1

$F_2$  distributions from three other green by white crosses are also shown in Table 2. Although the magnitude of the deviations from expectation in these populations is not large, the deviations are all in the same direction, as in cross 63.

The results of a small field planting of these  $F_2$ s, for which data is not presented, further indicate that the factorial situation in the parents involved is similar to that in the Little Dutch  $\times$  White Burley cross.

EVIDENCE FROM THE  $F_3$  GENERATION

Greenhouse tests of 108  $F_3$  progenies from cross 63 resulted in the distribution with regard to segregating and nonsegregating families shown in Table 3.

TABLE 3—Greenhouse counts on 108  $F_3$  progenies from cross 63

Ratio	All green	15 green 1 white	3 green 1 white
Observed.....	53	31	24
Expected, on basis of dihybrid segregation.....	50.4	28.8	28.8

$$\chi^2=1.10215 \quad P=0.75$$

Attention was next devoted to a series of controlled experiments in which counted numbers of seed were used and a special effort was made to keep the moisture content of the soil at the optimum for germination. Since, in transplanting, only seedlings that had attained a certain size were removed from the stock flats at a given time, record was kept of successive transplantings to give further check on the possibility of a differential growth rate between green and white seedlings.

The results of such an experiment are shown in Table 7. One thousand seeds of each of six progenies were sown on greenhouse flats, and all plants were tested. The data show no evidence of differential growth rate between green and white seedlings.

TABLE 7.—Greenhouse counts on  $F_2$ ,  $F_3$ , and back crossed  $F_1$  of Little Dutch  $\times$  White Burley (cross 63)

[Analysis of successive transplantings from 1,000 seed sown on stock flats to show effect of selection in transplanting on percentage of whites recovered from segregating progenies]

Progeny No	Transplanting No	Plants			Deviation from ratio indicated	Dev P E
		Green	White			
		Number	Number	Per cent		
C63F <sub>2</sub> -----	{ 1	249	21	7.8	4.1 from 15.1	1.5
	2	253	23	8.3	5.8 from 15.1	2.1
	3	95	6	5.9	—3 from 15.1	2
Total-----		597	50	7.7	9.6±4.2 from 15.1	2.3
C63F <sub>1</sub> × White Burley-----	{ 1	160	47	22.7	—4.8 from 3.1	1.1
	2	27	5	15.6	—3.0 from 3.1	1.7
Total-----		187	52	21.8	—7.8±4.5 from 3.1	1.7
63-24 (F <sub>3</sub> )-----	{ 1	156	53	22.2	—6.8 from 3.1	1.5
	2	147	39	21.0	—7.5 from 3.1	1.9
	3	148	61	29.2	8.8 from 3.1	2.1
	4	89	29	24.6	—5 from 3.1	1
Total-----		570	182	24.2	—6.0±6.7 from 3.1	9
63-26-----	{ 1	191	62	24.5	—3 from 3.1	1
	2	161	59	26.8	4.0 from 3.1	9
	3	43	14	24.6	—3 from 3.1	1
Total-----		395	135	25.5	2.5±6.7 from 3.1	4
63-28-----	{ 1	262	20	7.1	2.4 from 15.1	9
	2	229	19	7.7	3.5 from 15.1	1.8
	3	141	9	6.0	—4.4 from 15.1	.2
	4	55	8	12.7	4.1 from 15.1	3.1
Total-----		687	56	7.5	9.6±4.5 from 15.1	2.1
63-34-----	{ 1	175	18	9.3	5.9 from 15.1	2.6
	2	269	29	9.7	10.4 from 15.1	3.7
	3	235	6	2.5	—9.1 from 15.1	3.6
	4	39	5	11.4	2.3 from 15.1	2.1
Total-----		718	58	7.5	9.5±4.6 from 15.1	2.1

In the second part of this experiment, which was intended as a check on the soil tests, seed was germinated on moist filter paper in Petri dishes to give each seed the maximum chance to produce a plant. After 10 days, the very young seedlings were transferred to soil by removing the filter paper, placing it on a leveled flat, and covering it with a thin layer of finely sifted soil. Since in the second week following this transfer many seedlings died, this test can not be considered in the light of a check on the same progenies germinated

TABLE 5—Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63), segregation in back cross to White Burley of  $F_2$  green selections which, on self pollination, yielded  $F_3$  progenies segregating for one and for two factors

Designation	Progenies	Plants				Ratio	Deviation	Dev P E
		Green		White				
63F <sub>2</sub> × WB	Number	Number	Number	Per cent				
	18	5,885	1,779	23.2	3.1	-137.0 ± 25.6	5.4	
	15	2,708	2,588	45.9	1.1	-60.0 ± 24.6	2.4	

#### INVESTIGATION OF THE CAUSE OF THE RECESSIVE DEFICIENCY

In an attempt to discover the factors responsible for the recessive deficiencies observed, and to determine if possible the stage at which recessives were discriminated against, the following investigations were undertaken.

It was thought that certain phases of the greenhouse technic might have influenced the percentage of whites recovered, particularly the discarding of plants remaining in stock flats after transplanting. This failure to test all the plants raised in stock flats was appreciable only in the first few months of the greenhouse tests, thereafter effort was made to sow a sufficiently small quantity of seed of each progeny to enable practically all the plants grown to be tested. An analysis of the records for certain highly deficient progenies was made by separating the first and last half of the populations transplanted from stock flats for comparison with regard to the percentage of recessives in each half. These data are presented in Table 6 and show no evidence of a differential growth rate between dominant green and recessive white seedlings. It is, therefore, reasonable to assume that no great error was introduced in the few cases in which the surplus stock plants were not tested.

TABLE 6—Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63), showing effect of selection on percentage of whites recovered in successive transplantings of segregating progenies from stock flats

F <sub>3</sub> PROGENIES RECESSIVE DEFICIENT FOR 3:1 RATIO							
Progeny No	Transplanted to—	Plants			Deviation	Dev P E	
		Green	White				
		Number	Number	Per cent			
63-22	Flats 1-8	767	194	20.2	-46.3 $\pm$ 9.1	5.1	
	Flats 9-16	774	186	19.4	-54.0 $\pm$ 9.1	5.9	
63-24	Flats 1-4	331	130	28.2	14.7 $\pm$ 6.3	2.3	
	Flats 5-8	391	89	18.5	-31.0 $\pm$ 6.4	4.8	
63-33	Flats 1-3	730	169	18.8	-55.8 $\pm$ 8.8	6.3	
	Flats 4-6	691	168	19.6	-46.8 $\pm$ 8.6	5.4	
63-38	Flats 1-4	966	275	22.2	-35.3 $\pm$ 10.3	3.4	
	Flats 5-9	1,150	341	22.9	-31.8 $\pm$ 11.3	2.8	
F <sub>2</sub> PROGENIES DEFICIENT FOR 15:1 RATIO							
B63F <sub>2</sub>	Flats 1-4	905	41	4.3	-18.1 $\pm$ 5.0	3.6	
	Flats 5-8	924	40	4.1	-20.3 $\pm$ 5.1	4.0	
H63F <sub>2</sub>	Flats 1-3	797	28	3.4	-23.6 $\pm$ 4.7	5.0	
	Flats 4-6	904	45	4.7	-14.3 $\pm$ 5.0	2.9	
J63F <sub>2</sub>	Flats 1-3	901	40	4.3	-18.8 $\pm$ 5.0	3.8	
	Flats 4-5	593	24	3.9	-14.6 $\pm$ 4.1	3.6	
K63F <sub>2</sub>	Flats 1-3	913	42	4.4	-17.7 $\pm$ 5.1	3.5	
	Flats 4-6	907	42	4.4	-17.3 $\pm$ 5.0	3.5	

That the percentages of white segregates were not greatly affected, however, is shown by a comparison of the totals of each group. The irregular behavior of the individual progenies within the three groups make any statistical interpretation a difficult procedure. There was, however, a slight increase in the percentages of recessives obtained as the thickness of sowing increased.

The results of the Petri dish transfers are given in Table 10. With individual back crosses the full quota of recessives has been recovered in four cases out of seven, which is a normal expectation. Although seedling mortality in these transfers was again rather high, the results, in comparison with those of the same progenies germinated in soil, indicate that the actual elimination of recessives takes place during germination, although the effect of the recessive genotype may be exerted to some extent earlier in the ontogeny of the seed.

TABLE 10—*Greenhouse counts on Little Dutch × White Burley (cross 63), when 500 seed of each back-crossed F<sub>2</sub> selection were germinated in Petri dishes and then transferred to soil*

Progeny No	Germination	Plants surviving transfer	Plants			Deviation from 1:1 ratio	Dev P E
			Green	White			
		Per cent	Number	Number	Per cent		
63-22×White Burley...	37	100	53	47	47.0	-3 ±3.4	0.9
63-24×White Burley...	57	160	80	80	50.0	0 ±3.9	5
63-25×White Burley...	76	132	68	64	48.5	-2 ±4.6	2
63-26×White Burley...	85	188	93	95	50.5	1 ±4.4	2
63-27×White Burley...	85	360	179	181	50.3	2 ±6.8	3
63-47×White Burley...	86	406	201	205	50.5	2 ±5.1	9
63-61×White Burley...	73	227	118	109	48.0	-4 ±3.4	4
Total.....	713	1,573	792	781	49.7	-7.5 ±13.4	4

#### EFFECT OF WEIGHT OF SEED

To test for the effect of the weight of the seed, seed of each of seven F<sub>3</sub> families was separated into two weight classes, heavy and light, and counted numbers of each were sown on soil and in Petri dishes. After germination the seedlings in Petri dishes were transplanted individually to soil and grown to sufficient size for etiolation. Final counts on these transfers listed in Tables 11 and 12 show a small difference in the percentages of recessives recovered from heavy and light seed sown in soil, and a slightly smaller difference between these two classes with the seed germinated in Petri dishes. Closer examination of these tables reveals that two of the individual progenies, namely, 63-24 and 63-26, yielded a slight excess of recessives in three of the four tests. They were recessive deficient only in the test of light seed germinated in Petri dishes.

Within the limits of this experiment the behavior of these progenies is characterized by a degree of consistency which, in comparison with earlier tests, seems too great to be attributed to chance alone.

By consulting the deviations observed with the Petri dish transfers in Table 12, it is found that the recessive deficiencies are distributed at random with regard to the germination percentages of the progenies. This is to be expected since in these tests comparatively weak seeds have a better chance to germinate. However, a similar comparison of the germination percentages in this table with the deviations observed

in soil. Nevertheless, the data shown in Table 8 are of interest because of their close agreement with theoretical expectation for the ratios involved. In view of the rigorous treatment to which the plants were subjected, such a result indicates that recessive elimination must be sought for during germination or at some earlier stage in the life cycle.

TABLE 8.—Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63), when 500 seed of each segregating progeny were germinated in Petri dishes and transferred to soil

Progeny No	Plants			Deviation from ratio indicated	Dev P E
	Green		White		
	Number	Number	Per cent		
C63F <sub>1</sub> $\times$ White Burley	86	28	24.6	-0.5 $\pm$ 3.1 from 3.1	0.2
63-22	73	24	24.7	-3.2 $\pm$ 2.9 from 3.1	1
63-24	78	25	24.3	-8.3 $\pm$ 3.0 from 3.1	3
63-26	73	27	27.0	2.0 $\pm$ 2.9 from 3.1	7
Total	310	104	25.1	5.5 $\pm$ 5.9 from 3.1	1
63-28	56	4	6.7	3.1 $\pm$ 3.3 from 15.1	2
63-34	263	14	5.1	-3.3 $\pm$ 2.7 from 15.1	1.2
Total	319	18	5.3	-3.1 $\pm$ 3.0 from 15.1	1.0

#### THE EFFECT OF THICKNESS OF SOWING

It seemed possible that the thickness of sowing might be one of the factors affecting the percentage of recessives germinating in segregating populations. In another experiment, therefore, seed of seven back-crossed progenies was sown at three different rates in partitioned greenhouse flats. Germination tests on additional seed of these progenies were run in Petri dishes, and the resulting seedlings were transferred to soil by "flooding off" with a fine-tipped wash bottle.

The results of the different rates of sowing in soil are presented in Table 9. It will be noted that the total number of plants yielded by the sowing on whole flats checks very closely with the total for the sowing on one-eighth flats, whereas from comparable seed sown on one-quarter flats only about half as many plants were obtained. Since the soil in these tests was thoroughly mixed before sterilization, this inconsistency is probably due to insufficient moisture during the crucial stages of germination, in spite of the care taken to prevent such an occurrence.

TABLE 9.—Greenhouse counts on Little Dutch  $\times$  White Burley (cross 63), showing effect of thickness of sowing on the green:white ratio obtained with 3,500 seed of back-crossed F<sub>2</sub> selections sown on different areas

Designation	Area sown	Plants			Deviation from 1:1 ratio	Dev P E
		Green		White		
		Number	Number	Per cent		
63F <sub>2</sub> $\times$ WB	Square inches					
	300	638	601	48.5	-18.5 $\pm$ 11.9	1.6
	75	314	287	48.6	-8.5 $\pm$ 8.3	1.0
	37.5	606	637	51.2	15.5 $\pm$ 11.9	1.3

it indicates that the different progenies contain variable percentages of weak seed which germinate in Petri dishes but do not germinate so successfully in soil. Evidently it is among the weaker seeds that recessive elimination occurs.

There remain to be presented germination experiments with mixtures of true-breeding green and white families. In the first of these seed of Little Dutch, the green parent in cross 63, was mixed with seed of 63-31, a White Burley type segregate from cross 63, and the mixture sown on soil in a greenhouse flat. After the seedlings were transplanted and etiolated in the usual manner, the percentage of white plants resulting from the mixed sowing was compared with that expected, using separate soil-germination tests of the two component lines as a basis for calculation. As is apparent from the data in Table 13 the relative germination percentage of the recessive White Burley type seed was markedly decreased by the conditions of mixed sowing.

TABLE 13—Greenhouse counts on a mixture of pure-line green and pure-line white seed sown in soil

Designation	Seed sown	Transplanted to flat No	Plants			Percentage green on basis of total plants obtained	Percentage white on basis of total plants obtained	Deviation	Dev P E
			Green	White					
	Number		Number	Number	Per cent			Per cent	
63-31 (white) Little Dutch	1,000		0	856			59.8		
	1,000		576	0		40.2			
63-31 + Little Dutch.	1,000+1,000	1	144	165	53.4	}			
		2	156	109	41.1				
		3	88	153	63.5				
		4	111	169	60.4				
		5	32	21	39.6				
Total			531	617	53.7	46.3	53.7	a-b 1±0.870	7.0

\* Expectation calculated on basis of respective germination of green and white checks sown separately

To test this point further, green and white stocks were made up from seed of true-breeding  $F_3$  progenies having germinative capacities more nearly equal, with the object of balancing out any physiological weakness present in seed of a single family. The composition of these stocks and their respective germination percentages in Petri dishes are given in Table 14.

TABLE 14—Composition of green and white stocks and germination of constituent progenies in Petri dishes

Designation of stock	Constituent progenies	Germination in 10 days	Average germination
		Per cent	Per cent
Green A	63-30	93	92
	63-32	92	
	63-36	90	
	63-31	97	
White A	63-225	92	95
	63-20	96	
	A232-21	85	
White B	A232-22	95	89
	A232-23	88	

in the soil tests shown in Table 11 reveals that in the light-seed class the two positive deviations were produced by 63-24 and 63-26, two progenies with germination percentages of 86 and 91 per cent, respectively. Also, 63-61, the only other progeny whose light seed tested over 80 per cent in Petri dishes, has a negligible deviation in the soil test of  $-0.5$

TABLE 11 —Greenhouse counts on *Little Dutch* × *White Burley* (cross 63), showing the effect of weight of seed on the green : white ratio obtained with 500 heavy and 500 light seed of segregating  $F_3$  progenies sown on one greenhouse flat

Light or heavy seed	Progeny No	Plants			Deviation from 3 : 1 ratio	Dev P E
		Green	White			
		Number	Number	Per cent		
Light	63-22	112	32	22.2	-4.0± 3.5	1.1
	63-24	178	61	25.5	1.3± 4.5	3
	63-25	139	34	19.7	-9.3± 3.8	2.4
	63-26	164	57	25.8	1.8± 4.3	4
	63-27	179	51	22.2	-6.5± 4.4	1.5
	63-47	72	11	13.3	-9.8± 2.7	3.6
	63-61	230	76	24.8	-5.5± 5.1	1.1
	Total	1,074	322	23.1	-27.0±10.9	2.5
Heavy	63-22	132	31	19.0	-9.8± 3.7	2.6
	63-24	241	88	26.7	5.8± 5.3	1.1
	63-25	245	69	22.0	-9.5± 5.2	1.8
	63-26	186	81	30.3	14.3± 4.8	3.0
	63-27	184	54	22.7	-5.5± 4.5	1.2
	63-47	144	40	21.7	-6.0± 4.0	1.5
	63-61	223	71	24.1	-2.5± 5.0	5
	Total	1,355	434	24.3	-13.3±12.4	1.1

TABLE 12 —Greenhouse counts on *Little Dutch* × *White Burley* (cross 63), showing the effect of weight of seed on the green : white ratio obtained with 300 heavy and 300 light seed of segregating progenies germinated in Petri dishes and then transferred to soil

Progeny No	Light or heavy seed	Average germination	Seedlings transferred	Seedlings surviving	Plants			Deviation from 3:1 ratio	Dev P/E
					Green	White			
		Per cent	Number	Number	Number	Number	Per cent		
63-22	Light-----	65	197	188	140	48	25.5	1.0± 4.0	0.3
63-24		86	258	252	192	60	23.8	-3.0± 4.6	7
63-25		77	232	219	158	61	27.9	6.3± 4.3	1.5
63-26		91	274	254	194	60	23.6	-3.5± 4.7	7
63-27		58	174	169	130	39	23.1	-3.3± 3.8	9
63-47		54	164	160	117	43	26.9	3.0± 3.7	8
63-61		95	287	276	216	60	21.7	-9.0± 4.9	1.8
Total--		-----		1,586	1,518	1,147	371	24.4	-8.5±11.4
63-22	Heavy-----	97	291	291	224	67	23.0	-5.8± 5.0	1.2
63-24		98	305	305	228	77	25.2	8.5± 5.1	2
63-25		97	290	259	198	61	23.6	-3.8± 4.7	8
63-26		99	297	297	216	81	27.3	6.8± 5.0	1.4
63-27		97	290	269	211	58	21.6	-9.3± 4.8	1.9
63-47		97	292	265	196	69	26.0	2.8± 4.8	6
63-61		97	292	279	206	73	26.2	3.3± 4.9	7
Total--		-----		2,057	1,965	1,479	486	24.7	-5.3±13.0

\* 310 seed instead of 300 used in this sample.

It is evident that progenies with the lowest germination percentages in Petri dishes are most deficient in recessives. The effect is somewhat less pronounced with the heavy seeds, but here 63-24 and 63-26 again produce a small excess of recessives. This behavior is significant since

## DISCUSSION

Considering the whole of the data presented on segregation in  $F_2$ ,  $F_3$ , and back crosses of  $F_1$ , and  $F_2$  of Little Dutch  $\times$  White Burley as well as the  $F_2$  results of other green by white crosses, the genetic behavior of the White Burley character is best explained on the basis of duplicate factors which may be designated  $G_1 g_1 G_2 g_2$ . Since  $F_2$  segregation in several other green by white crosses closely parallels that observed in Little Dutch  $\times$  White Burley it is probable that green and White Burley varieties commonly differ in these same two factor pairs.

On the basis of two independent duplicate Mendelian factors one would expect to recover in  $F_2$  6.25 per cent of the recessive white genotype  $g_1 g_1 g_2 g_2$ . Normally the deviations from this theoretical percentage should be equally distributed as to direction. That this expectation is not realized with progenies of Little Dutch  $\times$  White Burley is evident from a consideration of almost any table of field or greenhouse results presented. The nature of the recessive deficiencies is clearly shown in Table 2, where segregation in 12  $F_2$  families grown in the greenhouse is recorded. It is equally apparent in Table 4, where small individual deficiencies lead to a total deficiency of over eight times the probable error.

That the recessive deficiencies are not caused by auxiliary genetic factors operating to modify the normal dihybrid ratio seems fairly clear. The similarity of reciprocal crosses and back crosses, and the contradictory behavior of certain  $F_2$  and  $F_3$  progenies in the field and in the greenhouse discourage attempts to account for the deficiencies on any such basis. Likewise, errors in classification and effect of selection in transplanting may be ruled out.

Since no deficiency of recessives was observed in the Petri dish transfers of several experiments, the actual elimination of recessives seems to occur during germination in soil. In this connection attention is again called to the physiological nature of the seed used in these experiments.  $F_3$  progenies have been shown to contain variable amounts of seed which will germinate in Petri dishes but not in soil. Furthermore, seed of high germination percentage in the Petri dish experiments yielded recessives in expected numbers, whereas seed of low germination percentage did not. Differential zygotic viability, therefore, between dominant green and recessive white seems to be the most logical explanation of the recessive deficient ratios observed. On this basis one would expect a progressive increase in the degree of recessive deficiency between sowings in Petri dishes, in greenhouse flats, and in exposed seed beds as the control of optimum conditions for germination is relaxed. Such an increase has been observed.

The work of Allard (1) on the aurea character in *Nicotiana rustica* is interesting in this connection. Segregation in the  $F_2$  generation of a green by aurea cross involving a total of 25,000 plants yielded 6,079 aureas, or 24.31 per cent. This total is apparently a summation of 13 separate  $F_2$  plantings, 11 of which showed small recessive deficiencies. The minus deviation from expected numbers in this total, however, is about 3.7 times its probable error. The total of the segregating  $F_3$  families and of the  $F_1$  back crossed to white-stemmed aurea also show small deficiencies in the recessive class. Appearance of chlorophyll-

Seed of the three stocks was sown separately and in mixtures at different rates on greenhouse flats. The results of this sowing, shown in Table 15, are somewhat contradictory. The green A stock yielded a significantly greater number of plants from 600 seeds sown on one-quarter flat than from an equal number of seeds sown on a whole flat, while the reverse is true of the white A and white B stocks. The low actual yield of plants in this particular test suggests the operation of some uncontrolled environmental factor during germination, an opinion supported by repetition of the check sowings at different rates as shown in the lower part of Table 16. Additional evidence that crowded sowing induces a higher yield of plants is also presented in Table 16. In this case the constituent progenies of the three stocks were tested separately and showed consistent increases in the number of plants obtained at the thicker sowing. A comparison of these data with those of the Petri dish germination tests shown in Table 14 reveals a congruity of behavior on the part of the stocks and their constituent progenies under the two conditions. There is no evidence in these tests that crowded sowing in soil results in discrimination against the recessive genotype.

TABLE 15.—Greenhouse counts on mixtures of green and white stocks sown on different areas in soil

Stock	Seed sown	Area sown	Plants obtained	Plants		
				Green	White	
	Number		Number	Number	Number	Per cent
Green-A-----	600	1 flat-----	145			
White-A-----	600	do-----	273			
White-B-----	600	do-----	236			
Green-A-----	600	$\frac{1}{4}$ flat-----	242			
White-A-----	600	do-----	146			
White-B-----	600	do-----	128			
Green-A+white-A-----	600+600	1 flat-----		260	232	47.2
Green-A+white-B-----	600+600	do-----		117	172	59.5
Green-A+white-A-----	600+600	$\frac{1}{4}$ flat-----		161	161	50.0
Green-A+white-B-----	600+600	do-----		145	142	49.5

TABLE 16.—Greenhouse counts on constituent progenies of green and white stocks from 600 seeds sown separately in soil

Stock	Constituent progenies	Area sown	Plants re-covered	Total plants per stock
			Number	
Green-A-----	63-30	1 flat-----	308	1,033
	63-32		108	
	63-36		317	
	63-31		169	
White-A-----	63-225	1 flat-----	283	1,172
	63-20		420	
	A 232-21		344	
White-B-----	A 232-22	1 flat-----	404	1,153
	A 232-23		405	
	63-30		436	
Green-A-----	63-32	1 flat-----	514	1,461
	63-36		511	
	63-31		557	
White-A-----	63-225	$\frac{1}{4}$ flat-----	472	1,508
	63-20		479	
	A 232-21		430	
White-B-----	A 232-22	1 flat-----	493	1,392
	A 232-23		469	
	63-30		352	
Green-A-----		$\frac{1}{2}$ flat-----	388	
White-A-----			411	
White-B-----			458	
Green-A-----		$\frac{1}{8}$ flat-----	516	
White-A-----			513	
White-B-----				

Experiments with heavy and with light seed germinated in soil and in Petri dishes demonstrate that segregating progenies normally contain variable amounts of weak seed which will germinate in Petri dishes but not in soil. Elimination of recessives in these experiments tends to decrease with an increase in relative viability of the light seed.

A differential viability between dominant green and recessive white genotypes is therefore apparently responsible for the recessive deficiencies observed.

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deficient recessives in less than expected numbers, therefore, seems to be characteristic of this species of *Nicotiana* as well

Similar phenomena have been reported by other investigators, particularly with maize, where the existence of many recessive abnormalities has been established. Lindstrom (?) studied a number of recessive chlorophyll modifications affecting the mature corn plant. In populations segregating for these characters slight recessive deficiencies were regularly encountered.

In tobacco it is probable that the degree of recessive deficiency is materially influenced by environmental conditions during seed development. It is conceivable that back-crossed seed involving the maturation of only a few capsules per plant would be physiologically stronger than self-fertilized seed from a plant maturing many capsules. Certain of the results obtained with self-fertilized and back-crossed seed of the same progenies might be explained on this basis.

### SUMMARY

The inheritance of the chlorophyll-deficient White Burley character in crosses of White Burley with normal green tobaccos has been studied in field plantings, in greenhouse plantings, and in transfers to soil of seed germinated in Petri dishes. A special technic for the identification of this character in greenhouse seedlings has been developed and was employed in the classification of segregating progenies.

Data from the  $F_1$ ,  $F_2$ , and  $F_3$  generations and from back crosses of  $F_1$  and  $F_2$  show that duplicate genes designated  $G_1$  and  $G_2$  are involved in the production of the normal green color of the plant. Their recessive allelomorphs  $g_1$  and  $g_2$  are both essential to the White Burley genotype.  $F_1$  plants are fully green, and segregation in  $F_2$  is accordingly 15 green:1 white. In  $F_3$  true-breeding green and true-breeding white progenies were recovered as well as families segregating for one and for two factors.

Large recessive deficiencies in field plantings, and smaller but equally distinct recessive deficiencies in greenhouse plantings were observed in all segregating generations. Evidence from successive greenhouse transplantings, and data from experiments involving counted numbers of seed show that selection from field seed beds and from greenhouse stock flats is not responsible for these deficiencies.

Experiments with counted numbers of seeds show that crowded sowing does not cause the elimination of recessives since frequently increases in the percentage of germination paralleled by slight increases in percentage of recessives recovered were obtained with thickly sown seed.

Evidence that environmental and not genetic factors are concerned in the nonappearance of recessives in expected numbers is fourfold: (1) No significant differences are observed in the  $F_2$  of reciprocal crosses and in reciprocal back crosses of the green  $F_1$  to White Burley; (2) the differences between field and greenhouse results are most logically explained on the basis of nongenetic influence; (3) certain  $F_3$  progenies produce an excess of recessives in some greenhouse experiments and a deficiency in others; and (4) in some Petri dish transfers normal expectation for recessives is realized.

# AN ADDITIONAL PAIR OF FACTORS AFFECTING ANTHOCYANIN PIGMENT IN MAIZE<sup>1</sup>

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## INTRODUCTION

This paper reports a new gene affecting the production of anthocyanin pigment in maize (*Zea mays* L.) This gene is so similar in its action, both in aleurone and in plant color, to the *Aa* factor pair reported by Emerson<sup>2</sup> in 1918 that it has been called *A<sub>2</sub>a<sub>2</sub>*. It is suggested that the *Aa* factor pair isolated by Emerson be designated *A<sub>1</sub>a<sub>1</sub>*.

## MATERIAL

The *A<sub>2</sub>a<sub>2</sub>* factor pair was obtained from Iodent corn, a strain of Reid Yellow dent developed at the Iowa Agricultural Experiment Station<sup>3</sup>. The original progeny heterozygous for *A<sub>2</sub>a<sub>2</sub>* also was heterozygous for the chlorophyll defect, *iojap*. In fact, the material was being used for a study of this chlorophyll defect, and the presence of *a<sub>2</sub>* was not suspected until peculiar ratios of aleurone colors were obtained. Many of the data presented in this paper have come from the F<sub>1</sub>, F<sub>2</sub>, and later generations of an individual cross between the *a<sub>2</sub>* stock and an *A<sub>1</sub>* tester for aleurone color which was homozygous for brown plant color. The genetic composition of this cross was as follows:

*a<sub>1</sub>a<sub>1</sub> A<sub>2</sub>A<sub>2</sub> CC RR BB Pl Pl p p* × *A<sub>1</sub>A<sub>1</sub> A<sub>2</sub>a<sub>2</sub> cc rr bb pl pl P<sup>wt</sup> P<sup>wt</sup>*

This cross will be referred to throughout as the original F<sub>1</sub> cross.

## INTERACTION OF *A<sub>2</sub>* WITH ALEURONE GENES *A<sub>1</sub>*, *C*, AND *R*

Aleurone color in maize is dependent upon the presence of the dominant allelomorphs of the three complementary pairs of genes *A<sub>1</sub>a<sub>1</sub>*, *Cc*, and *Rr* and the duplex recessive condition of the inhibitor, *Ii*. The interactions of these genes are so familiar to every student of maize genetics that they need not be reviewed here. In its effect on aleurone color the new gene, *A<sub>2</sub>*, is complementary to the three complementary genes mentioned above.

Fifty-five of the plants of the original F<sub>1</sub> cross were self-pollinated and yielded F<sub>2</sub> progenies. Twenty-four of these progenies segregated for aleurone color in the proportion of 27 colored to 37 colorless, and

<sup>1</sup> Received for publication Nov. 2, 1931, issued May, 1932. The data on which this paper is based come from the corn-improvement project conducted cooperatively by the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Farm Crops Section of the Iowa Agricultural Experiment Station.

<sup>2</sup> EMERSON, R. A. A FIFTH PAIR OF FACTORS, *AA*, FOR ALEURONE COLOR IN MAIZE, AND ITS RELATION TO THE *CC* AND *RR* PAIRS. N. Y. Cornell Agr. Expt. Sta. Mem. 16, p. 227-289, illus. 1918.

<sup>3</sup> The writer is informed by G. W. Beadle, of the New York State College of Agriculture, Ithaca, N. Y., that he isolated *a<sub>2</sub>* from some Argentine flint corn collected by F. D. Richey and R. A. Emerson at Casilda, Argentina.



A summary of the dihybrid segregations among these  $F_4$  progenies which involved  $A_2a_2$  and each of the other complementary factors for aleurone color is recorded in Table 4.

TABLE 4.—Summary of  $F_4$  dihybrid segregations involving  $A_2a_2$  and each of the other complementary genes for aleurone color

Genes heterozygous	Number of progenies	Observed		Expected		Dev P E
		Colored	Colorless	Colored	Colorless	
$A_2a_2$ and $A_1a_1$ .....	20	4,339	3,481	4,399	3,421	2 0
$A_2a_2$ and $Cc$ .....	7	1,379	1,061	1,373	1,067	.4
$A_2a_2$ and $Rr$ .....	9	1,618	1,290	1,636	1,272	1 0

Frequency distributions of the aleurone color segregations obtained in the  $F_2$ ,  $F_3$ , and  $F_4$  progenies, summarized in Tables 2 and 3,

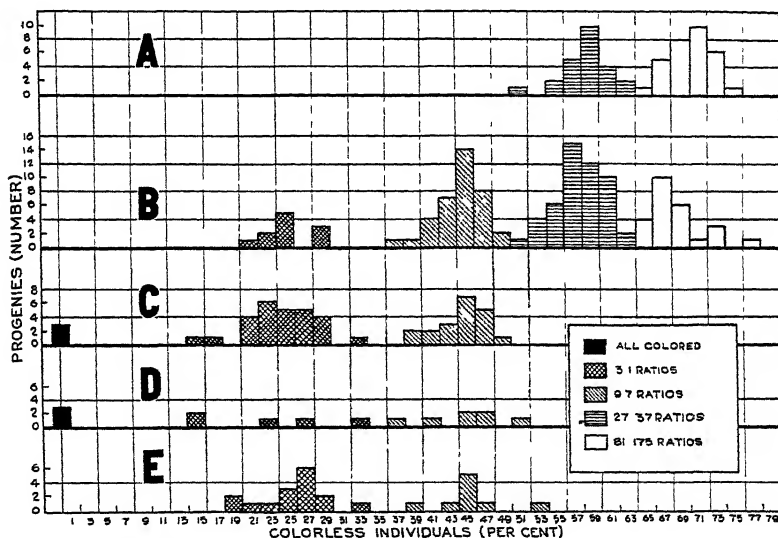


FIGURE 1.—Frequency distributions of aleurone color. A,  $F_2$  progenies, B,  $F_3$  progenies from plants produced by colored seeds of 81 175 progenies from A, C, D, and E,  $F_4$  progenies from plants produced by the colored seeds of 97 progenies from B. C is from the 97 progenies heterozygous for  $A_1a_1$  and  $A_2a_2$ , D from those heterozygous for  $A_2a_2$  and  $Cc$ , and E from those heterozygous for  $A_2a_2$  and  $Rr$ .

are shown in Figure 1. It will be seen that there was some overlapping of the different kinds of ratios. This overlapping, however, does not appear to be of sufficient extent to discredit the progeny classifications presented in Tables 2 and 3.

The data presented in this section indicate that  $A_2$  is complementary to  $A_1$ ,  $C$ , and  $R$  in the production of aleurone color. They also indicate that  $A_2$  is inherited independently of these three genes. More complete proof, however, that  $A_2$  is not linked in inheritance with  $A_1$ ,  $C$ , or  $R$  is presented in the section on linkage.

the remaining 31  $F_2$  progenies segregated for aleurone color in proportions approximating 81 colored to 175 colorless. Equal numbers of each kind of segregation were expected. The deviation of 3.5 is 1.4 times its probable error. A summary of the data from these 55 progenies is recorded in Table 1.

TABLE 1.—Summary of data on 55  $F_2$  progenies

Ratio	Number of progenies	Colored		Colorless		Dev P E
		Observed	Expected	Observed	Expected	
27:37.....	24	4,622	4,693	6,501	6,430	0.67
51:175.....	31	4,278	4,539	10,067	9,806	2.18

Six of the  $F_2$  progenies showing aleurone color ratios of 81:175 were grown in the field, and a large number of the plants from colored seeds were self-pollinated. A summary of the aleurone color segregations of the 123  $F_3$  progenies obtained is recorded in Table 2.

TABLE 2.—Data on  $F_3$  aleurone color segregations obtained by selfing  $F_2$  plants produced by colored seeds from 81:175 ratios

Aleurone ratio	Number of $F_3$ progenies		Deviation
	Observed	Expected	
All colored.....	0	1.5	-1.5
3 colored to 1 colorless.....	11	12.2	-1.2
9 colored to 7 colorless.....	37	36.5	+ .5
27 colored to 37 colorless.....	50	48.6	+1.4
51 colored to 175 colorless.....	25	24.3	+ .7
Total.....	123	123.1	- .1

$$\chi^2 = 1.69 \quad P = 0.8-$$

A number of the 9:7  $F_3$  progenies were grown in the field and tested to determine which of the aleurone factors were heterozygous. Among those grown there were some heterozygous for  $A_2a_2$  and each of the factor pairs  $A_1a_1$ ,  $Cc$ , and  $Rr$ . Plants from the colored seeds in these progenies were self-pollinated. They yielded  $F_4$  progenies, of which some were homozygous colored, some segregated in ratios of 3 colored to 1 colorless, and some segregated for 9 colored to 7 colorless. A summary of the number of progenies showing each kind of segregation is recorded in Table 3.

TABLE 3.—Summary of  $F_4$  aleurone color segregations obtained by selfing  $F_3$  plants produced by the colored seeds from the dihybrid ratios involving  $A_2a_2$  and each of the other complementary genes for aleurone color

Genes heterozygous	Number of progenies giving aleurone ratios indicated						Total
	All colored		3 colored to 1 colorless		9 colored to 7 colorless		
	Observed	Expected	Observed	Expected	Observed	Expected	
$A_{1a_2}$ and $A_{1a_1}$ .....	3	5.6	27	22.2	20	22.2	50
$A_{2a_2}$ and $Cc$ .....	4	1.8	5	7.1	7	7.1	16
$A_{2a_2}$ and $Rr$ .....	0	2.8	16	11.1	9	11.1	25

INTERACTION OF  $A_2$  WITH PERICARP GENE  $P^{wr}$ 

The interaction of factors in the production of the red series of pericarp colors has been shown by Emerson<sup>5</sup> and by Anderson and Emerson<sup>6</sup> to be as follows.

$A_1$   $P$ =red.  
 $a_1$   $P$ =brown.  
 $A_1$   $p$ =colorless.  
 $a_1$   $p$ =colorless.

The original  $F_1$  cross was heterozygous for  $P^{wr}$  (white pericarp, red cob) and  $p$ . The presence of brown plants with red cobs in the  $F_2$  progenies segregating for  $A_2a_2$  indicated that recessive  $a_2$  must not affect pericarp and cob color in the same manner as recessive  $a_1$ . The  $F_2$  distributions obtained, however, were not in agreement with those expected on the assumption that  $A_2a_2$  had no influence whatever upon cob color. An excess of brown plants with brown cobs was obtained which could not be accounted for. During the past season several progenies were grown which were segregating for  $A_2a_2$  and  $P^{wr}p$  and were homozygous for  $A_1A_1$ . Only red and white cobs were expected in these progenies. The unexpected occurrence of brown plants with brown cobs led to a more careful examination of the colored portions of these cobs. It developed that the color in these exceptional cobs was due to brown color located in the horny basal portion of the lower glumes and that the thin upper glumes or chaff were white.

In the mature pistillate spikelet of maize the lower (empty) glumes are thick and horny one-half to three-quarters of the distance from base to tip. They usually have thin membranous margins varying greatly in width. The upper glumes (lemmas and paleas) are thin and membranous.

The color produced by the pericarp gene  $P^{wr}$  appears to be confined to the thin upper glumes and to the thin margins of the lower glumes. The color of these parts will be referred to as upper glume or chaff color.

The color of the horny portions of the lower glumes (which will be termed lower glume color) is associated with plant color. In the cultures used in this study the basic color of the lower glumes on the ears of dilute sun red plants was light yellow or straw color. The color of these glumes was modified by the plant color factors and was correlated with plant color as follows.

Plant color	Lower glume color
Purple=purple	
Sun red=straw	
Dilute purple=straw, occasionally showing considerable purple.	
Dilute sun red=straw	
Brown=brown.	
Green=straw.	

These colors doubtless vary somewhat in intensity in different cultures. Purple and dilute purple plants from different cultures show considerable variation in the amount of purple in the lower glumes, and brown plants in the amount of brown color. There also is much difference in the size of both upper and lower glumes in different cul-

<sup>5</sup> EMERSON, R. A. Op cit. (See footnote 4)

<sup>6</sup> ANDERSON, E. G., and EMERSON, R. A. PERICARP STUDIES IN MAIZE I. THE INHERITANCE OF PERICARP COLORS. Genetics 8 [466]-476 1923

INTERACTION OF  $A_2$  WITH PLANT COLOR GENES  $A_1$ ,  $B$ , AND  $Pl$ 

The interaction of the  $A_1$ ,  $B$ , and  $Pl$  factors has been reported by Emerson (1921)<sup>4</sup>. Gene  $A_2$  is complementary to  $A_1$  in its action upon plant color as well as in its action upon aleurone color. The  $F_1$  plants of the original previously mentioned cross, which were heterozygous for  $A_2a_2$ , were of the constitution  $a_1A_2 C R B Pl A_1a_2 c r b pl$ . The  $F_2$  progenies contained seedlings with red and with green stems in the ratio of 9 red to 7 green. Six  $F_2$  progenies were grown to maturity. The  $F_2$  plant color segregations obtained and those expected on the assumption that  $A_2$  is complementary to  $A_1$  are indicated in Table 5.

TABLE 5— $F_2$  plant color segregations

Genotypes		Phenotypes	Observed	Expected	Deviation
Composition	Number				
$A_1 A_1 B Pl$	81	Purple.....	306	306	0
$A_1 A_1 B pl$	27	Sun red.....	100	102	-2
$A_1 A_1 b Pl$	27	Dilute purple.....	93	102	-9
$A_1 A_1 b pl$	9	Dilute sun red.....	26	34	-8
$A_1 a_2 B Pl$	27	Brown.....	255	238	+17
$A_1 a_2 B pl$	27				
$A_1 a_2 b Pl$	9				
$A_1 a_2 b pl$	9				
$A_1 a_2 B Pl$	9				
$A_1 a_2 B pl$	9	Green.....	187	185	+2
$A_1 a_2 b Pl$	9				
$A_1 a_2 b pl$	3				
$A_1 a_2 B pl$	3				
$A_1 a_2 b Pl$	3				
$A_1 a_2 b pl$	1				
Total.....	256		967	967	0

$$\chi^2 = 3.98 \quad P = 0.5+$$

The greatest differences between the observed and expected numbers occur in the dilute purple, dilute sun red, and brown classes. The differences suggest a loose linkage, in the coupling phase, between  $A_2$  and  $B$ .

$F_2$  progenies were grown from a number of self-pollinated  $F_2$  plants. A summary of the  $F_3$  plant color segregations in the progenies from some of the  $F_2$  plants heterozygous for  $A_2a_2$  is recorded in Table 6.

All of these segregations are in agreement with the hypothesis that  $A_2a_2$  is complementary to  $A_1a_1$  in its action on plant color.

TABLE 6—Data on the plant color segregations in  $F_3$  progenies from  $F_2$  plants which were heterozygous for  $A_2a_2$ 

Group	Genetic composition	Pedigree	Purple	Sun red	Dilute purple	Dilute sun red	Brown	Green	Total
1 ( $A_1 A_1$ )	$A_1 A_1 A_2 a_2 BB Pl Pl$	5710	42				19		61
	Expected.....		46				15		61
	$A_1 A_1 A_2 a_2 BB Pl pl$	5706	40	12			9	2	63
	Expected.....		36	12			12	4	64
	$A_1 A_1 A_2 a_2 Bb Pl Pl$	5714	41		12		10	5	68
	Expected.....		38		13		13	4	68
	$A_1 A_1 A_2 a_2 Bb Pl pl$	5722 & 8			65	18		36	119
	Expected.....				67	22		30	119
	$A_1 a_1 A_2 a_2 BB Pl pl$	5690 & 4	49	20			29	17	115
	Expected.....		49	16			38	13	116
2 ( $A_1 a_1$ )	$A_1 a_1 A_2 a_2 Bb Pl Pl$	5696	22		9		22	2	55
	Expected.....		23		8		18	6	55
	$A_1 a_1 A_2 a_2 Bb pl pl$	5700		25		4		26	55
	Expected.....			23		5		24	55
	$A_1 a_1 A_2 a_2 Bb pl pl$	5702		21		10		24	55
	Expected.....			23		8		24	55

<sup>4</sup> EMERSON, R. A. THE GENETIC RELATIONS OF PLANT COLORS IN MAIZE. N. Y. Cornell Agr. Expt. Sta. Mem. 39, 1-156 pp., illus. 1921.

A summary of the factorial relations of  $A_1$ ,  $A_2$ , and  $P^{wr}$  in regard to upper glume color is as follows:

$$\begin{array}{l} A_1 A_2 P^{wr} \} \text{red} \\ A_1 a_2 P^{wr} \} \\ a_1 A_2 P^{wr} \} \text{brown} \\ a_1 a_2 P^{wr} \} \\ A_1 A_2 p \} \\ A_1 a_2 p \} \text{white or colorless} \\ a_1 A_2 p \} \\ a_1 a_2 p \} \end{array}$$

Data supporting the foregoing relationship were obtained from some of the  $F_3$  progenies. A summary of the data from two progenies segregating for these three factors is recorded in Table 8. The observed and expected numbers are not in very good agreement, but all of the expected classes are present.

TABLE 8—Data on two progenies by self-fertilization from plants of the composition  $A_1 a_1 A_2 a_2 P^{wr} p$

Item	Colored plants ( $A_1 A_2$ )		Green plants ( $a_1 A_2$ , $A_1 a_2$ , and $a_1 a_2$ )			Total
	Red chaff ( $P^{wr}$ )	White chaff ( $p$ )	Red chaff ( $P^{wr}$ )	Brown chaff ( $P^{wr}$ )	White chaff ( $p$ )	
Observed.....	30	20	13	23	7	93
Expected.....	39 2	13 1	13 1	17 4	10 2	93
Deviation.....	-9 2	+6 9	- 1	+5 6	-3 2	0

$$\chi^2=8.59 \quad P=\text{between } 0.05 \text{ and } 0.10$$

TABLE 9—Data on a progeny by self-fertilization from a plant of the composition  $A_1 a_1 A_2 a_2 P^{wr} P^{wr}$

Item	$A_1 A_2$ (red chaff) <sup>a</sup>	$A_1 a_2$ (red chaff)	$a_1 A_2$ and $a_1 a_2$ (brown chaff)	Total
Observed.....	25	10	12	47
Expected.....	27	9	12	48
Deviation.....	-2	+1	-0	-1

<sup>a</sup> Also includes the purple plants with purple chaff

A single  $F_3$  progeny was obtained which was segregating for  $A_1 a_1 A_2 a_2$  and was homozygous  $P^{wr} P^{wr}$ . A summary of the data from this progeny is recorded in Table 9. The agreement between the observed and expected numbers is very close in this case.

#### LINKAGE RELATIONS OF $A_2 a_2$

Crosses have been made between  $a_2$  and genes from 7 of the 10 linkage groups in maize. The linkage data from these crosses are summarized in Table 10.

Recombination values approximating 50 per cent were obtained between  $a_2$  and all the factors with which it has been crossed except  $B$ . From the back-cross data on  $a_2$  and  $B$ , crossover percentages of from 35.5 to 40.2 per cent may be computed, depending upon the

tures and consequently in their relative importance in determining the superficial color of the cob.

The basic colors of the upper glumes in the material studied were red and white. These colors were modified considerably on purple, dilute purple, and brown plants by the extension of purple and brown pigment into them. In the material used no difficulty was experienced, however, in determining whether their basic color was red or white, except in a few of the purple plants.

Gene  $a_2$ , unlike gene  $a_1$ , has no influence upon the upper glume color produced by the pericarp gene  $P$ . It influences lower glume color, however, in a manner exactly similar to  $a_1$ . On a brown plant with a red cob ( $A_1 a_2 B Pl P^{wr}$ ), therefore, the horny portions of the empty glumes were brown and the flowering glumes were red. The thin margins of the empty glumes were difficult to classify, but they appeared to be red similar to the flowering glumes.

A summary of the factorial relations of  $a_2$  and  $p$  in relation to upper glume or chaff color is as follows:

$$\begin{aligned} A_2 P^{wr} &= \text{red chaff} \\ a_2 P^{wr} &= \text{red chaff} \\ A_2 p &= \text{white chaff.} \\ a_2 p &= \text{white chaff.} \end{aligned}$$

Table 7 gives the data on four back-cross progenies which bear out this relationship. Inasmuch as these progenies also were back crosses for  $Pl pl$ , and as unfortunately the upper glume color of some of the purple plants could not be definitely established, it was necessary to group all the purple plants into one class.

TABLE 7—Data on progenies of the back cross  $\frac{a_2 P^{wr}}{A_2 p} \times a_2 p$

Pedigree No	Purple plants ( $A_2$ )	Sun-red plants ( $A_2$ )		Brown plants ( $a_2$ )		Green plants ( $a_2$ )	
		$P^{wr}$	$p$	$P^{wr}$	$p$	$P^{wr}$	$p$
7646.....	20	14	8	4	6	8	9
7649.....	11	10	9	6	12	8	11
7850.....	20	15	10	9	11	6	14
7851.....	14	13	13	8	6	8	4
Total.....	65	52	40	27	35	30	38
Expected.....	72	36	36	36	36	36	36
Deviation.....	-7	+16	+4	-9	-1	-6	+2

$\chi^2=11.62$   $P$ =between 0.05 and 0.10.

Among the  $F_2$  progenies grown there were a few that were homozygous  $P^{wr} P^{wr} A_1 A_1$  and that were demonstrated by appropriate tests to be segregating for  $A_2 a_2$ . All the plants in these progenies had red upper glumes on their cobs (with the exception, of course, of the purple plants, which had considerable purple pigment in these parts). These segregations support the assumption that the upper glume color of  $a_2 P^{wr}$  plants is red.

# THRESHER INJURY IN BABY LIMA BEANS<sup>1</sup>

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## INTRODUCTION

In the course of some germination studies of baby Lima beans, certain lots were found to produce rather high percentages of defective seedlings. The defects were of several different types, more than one of which frequently occurred in a single seedling. Of these various types of injury, one of the most conspicuous in the field when the beans are coming up is baldhead (fig 2, D), a condition in which the stem growing point and the first true leaves of the plant are missing. Harter,<sup>2</sup> in a rather thorough study of the causes of this condition in many varieties of beans, found that, while insects and bacteria are partly responsible for the trouble, by far the greater amount is caused by the threshing machine. He also found that varieties differ considerably in the degree to which they may be injured by the thresher, Lima beans being rather susceptible. Harter did not mention any type of machine injury, however, other than baldhead. This paper will, therefore, describe in some detail these other types of thresher injury found in Lima beans.

## TYPES OF INJURY

A study of many hundreds of baby Lima bean seedlings from machine-threshed seed shows that practically every part of the embryo is susceptible to some kind of thresher injury. In the following paragraphs, the various types of injuries are grouped according to the part of the plant affected.

### INJURIES TO COTYLEDONS

Detachment of the cotyledons is one of the most common injuries. One cotyledon may be broken at its point of attachment to the stem, the other functioning normally (fig 1, B), or both cotyledons may be absent although the plumule is uninjured (fig. 2, B and C). In either event, growth of the seedlings is retarded, although the retardation is much more pronounced in the latter than in the former case.

In many cases the cotyledon is not broken completely from the plant, but remains attached by a small area of tissue. Callus tissue develops at the broken surfaces, and not infrequently adventitious roots arise from this callused area of the cotyledon. In several cases cotyledons which had been broken off completely were found to have produced callus tissue and adventitious roots at their injured surfaces (Fig. 3, B and C.) One cotyledon of this type was transplanted to a pot and grown for a few weeks. During this time the cotyledonary bud, which apparently in this case was broken off with the cotyledon, produced a branch an inch or more long, the roots made a vigorous growth, and the cotyledon was almost completely absorbed.

<sup>1</sup> Received for publication Oct 5, 1931, issued May, 1932.

<sup>2</sup> HARTER, L. L. THRESHER INJURY A CAUSE OF BALDHEAD IN BEANS. *Jour Agr Research* 40 371-384, illus 1930.

method used. The value of  $37.2 \pm 3.2$  was computed from the first two classes. Gene  $a_2$  also has been crossed with  $lq$ , and the small amount of  $F_2$  data available indicate  $55 \pm 4.6$  per cent of recombinations. These data come from three small progenies which, because of the extremely dry season of 1930, were all that were obtained.<sup>7</sup>

TABLE 10—Recombination percentages of  $a_2$  with the genes with which it has been crossed

Genes		Linkage phase <sup>a</sup>	X Y	x Y	X y	x y	Total	Recombinations
X	Y							
1.	C	C Bc	4,666		14,422		19,088	<sup>b</sup> 51 $1 \pm 0.3$
1.	R	C Bc	1,802		5,763		7,565	<sup>b</sup> 52 $4 \pm 6$
1.	1.	C Bc	64	38		112	214	37 $2 \pm 3.2$
1.	Lg	R F <sub>2</sub>	92	35	74	34	235	55 $0 \pm 4.6$
1.	Y	R Bc	617	631	773	635	2,456	51 $0 \pm 7$
1.	Pl	C Bc	118	123	124	93	458	53 $9 \pm 1.6$
1.	T <sub>1</sub>	R Bc	109	123	117	132	481	50 $1 \pm 1.5$
1.	Br	R Bc	102	104	95	98	399	50 $1 \pm 1.7$
1.	Ra	R Bc	231	359	298	359	1,247	47 $3 \pm 1.0$
1.	Ij	C Bc	428	461	432	442	1,763	50 $7 \pm 8$
1.	A <sub>1</sub>	R Bc	6,445		19,536		25,981	<sup>b</sup> 49 $6 \pm 3$
1.	A	C Bc	1,692		5,171		6,863	<sup>b</sup> 50 $7 \pm 6$

<sup>a</sup> The symbols in this column have the following significance: C=coupling, R=repulsion, Bc=back-cross progenies, and F<sub>2</sub>=progenies by self-fertilization.

<sup>b</sup> Inasmuch as the recombination percentages in these distributions are computed from half of the total number of individuals, their probable errors also are based on the smaller numbers.

<sup>c</sup> A<sub>1</sub>a<sub>2</sub> also segregating.

## SUMMARY

A second pair of genes,  $A_2a_2$ , affecting anthocyanin pigment in maize has been isolated and its relation to some of the other aleurone and plant color genes studied.

The new factor pair is complementary to the  $A_1a_1$ ,  $Cc$ , and  $Rr$  pairs in the production of aleurone color.

It is complementary to  $A_1a_1$  in the production of plant color.

It differs from the  $A_1a_1$  pair only in that it has no influence upon the color produced by the pericarp gene  $P$ .

Cob color appears to be dependent upon the color of two distinct parts of the cob, the lower glumes and the upper glumes.

The color of the lower glumes appears to be controlled by the plant color genes and not influenced by the pericarp gene,  $P$ . The color of the thin upper glumes or chaff is influenced by  $P$ , and it is the color of these parts that is not influenced by  $A_2a_2$ .

<sup>7</sup> Linkage tests conducted since this was written of  $a_2$  with  $lq$ ,  $gl_2$ , and  $n_4$  indicate that  $a_2$  is not in this group.

soon replace it (fig 3, D), the growth of the seedling is retarded in consequence of the injury. Damage to the radicle usually results in pronounced curvature near the point of injury. (Fig. 4, C, D, and E)

A break in the hypocotyl frequently occurs just below the cotyledons, as in Figure 4, A and B. In this event the cotyledons remain below ground, while the first vegetative leaves reach the surface as a result of the elongation of the epicotyl. In these cases, adventitious roots also arise from the injured surface of the hypocotyl. Such seedlings are slow in coming up, and growers very often believe that they are from seeds which failed to get wet at the first irrigation.

A more common type of injury to the hypocotyl or root is one in which the fracture does not extend completely across it (Fig 2, A, C, and D). In a very large percentage of cases in which the hypocotyl

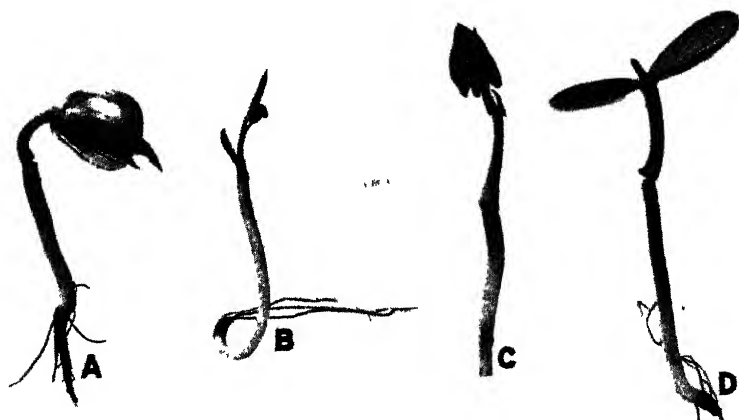


FIGURE 2—Lima bean seedlings showing various types of injury. A, Crack in the hypocotyl, B, both cotyledons and the radicle missing, C, both cotyledons missing, and hypocotyl cracked, D, baldhead bean with cracked hypocotyl

is injured in this manner, the injury is on the side of the hypocotyl away from the seed coat. This condition is well shown in Figure 2, A, and also in Figure 5. The explanation of this phenomenon appears to be connected with the way in which the hypocotyl and root are supported in the seed. The root tip is completely enveloped by seed-coat tissue adjacent to the micropyle, which gives it rather rigid support, while the opposite end of the hypocotyl is supported by its attachment to the cotyledons. The middle part of the hypocotyl in a baby Lima bean lies along the edge of the bean, adjacent to the crack between the two cotyledons. Although it usually lies against the cotyledons, it is not very firmly supported by them. As this axis is well supported at each end and not in the middle, a blow on the hypocotyl, such as might be delivered by a cylinder tooth in a threshing machine, might reasonably be expected to result in a bending of this structure inward at that point and in the opening of a crack on the side of the hypocotyl opposite that which received the impact.

Sometimes the fracture is of such a nature that, while the cotyledon still remains attached to the plant, its position with respect to the other cotyledon is altered. Such a situation is shown in Figures 1, A, and 4, C. One notes in the former case that the upper cotyledon, which is in its normal position, appears to be somewhat withered, while the lower one is still plump. In the latter case the opposite condition occurs, the upper cotyledon having been displaced. A break in the vascular connection of the plump cotyledon in each case probably explains why it has not lost its food reserve so rapidly as has the other one. Similar evidence appears in Figure 4, D, where a



FIGURE 1.—Lima bean seedlings showing injury to the cotyledons. A, Cotyledons at different levels as a result of injury near their point of attachment, the upper cotyledon being in its normal position, B, seedling with one cotyledon missing

cotyledon is shown with a crack crossing it transversely. Although the outer end is still attached, its food reserve has not been used as has that of the lower part of the cotyledon.

#### INJURIES TO HYPOCOTYL AND RADICLE

Besides the various injuries to the cotyledons just described, the hypocotyl and radicle are also subject to injury. Complete loss of the radicle is of comparatively frequent occurrence. (Fig. 4, C, D, and E.) In these cases the lower end of the hypocotyl calluses over and gives rise to adventitious roots. The radicle is occasionally found still attached to the lower end of the hypocotyl but injured to such an extent that it fails to grow. Although adventitious roots



FIGURE 4—Injuries to hypocotyl and radicle. A and B, Hypocotyl broken off at point of attachment of cotyledons, the hypocotyl and radicle being still present but not functioning in A, C, baldhead seedling with cotyledons at different elevations as a result of injury and with radicle missing, D, seedling with a fracture in one cotyledon, a crack across the hypocotyl, and the radicle missing, E, seedling with an injury to the hypocotyl near point of attachment of cotyledons, a transverse crack near the lower end of the hypocotyl, and radicle missing. Note the formation of adventitious roots at all injured surfaces.

Injuries of the type just described are not so serious in their subsequent effect on the plant as are some of the others mentioned previously. In most cases healing of these cracks in the hypocotyl begins very shortly after the bean commences to germinate, and, by the time the plant has two fully formed true leaves, the wound is

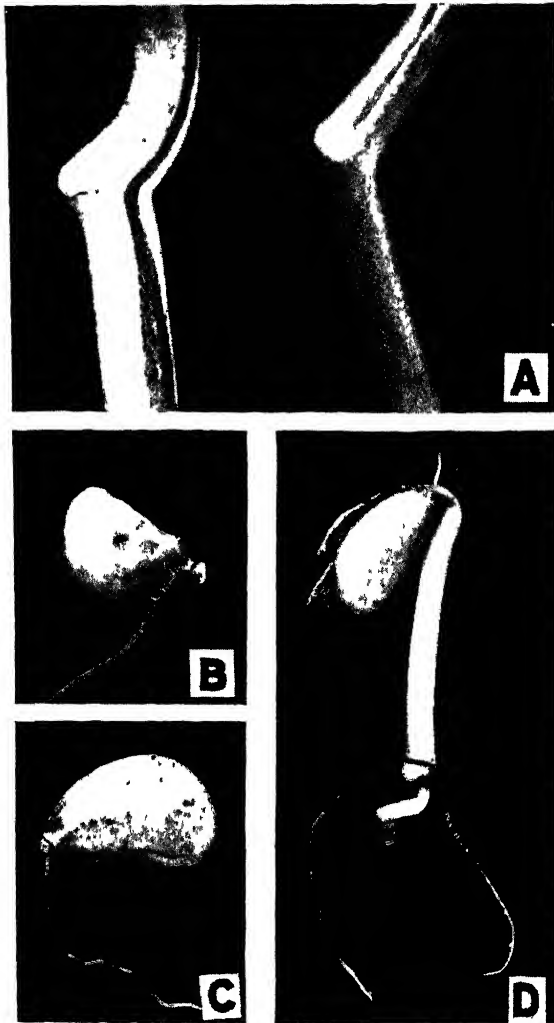


FIGURE 3 —Lima bean seedlings showing various types of injury. A, Cracked hypocotyls showing healing of the injury, B and C, detached cotyledons which have produced adventitious root from the wounded surface, D, seedling with injured radicle on which adventitious roots are being produced at the injured surface

completely healed. Early stages of such injuries are shown in Figure 5. These two embryos were taken from beans which had been soaked overnight. In later stages there is usually a sharp bend at the position of the injury, with an area of cork tissue forming a scar over the wound (Fig. 3, A.)

beans were germinated in soil flats in the manner already described. While a few cases of baldhead and of loss of cotyledons were observed, not a single seedling showed any injury to the hypocotyl or radicle.

In order further to establish the fact that the abnormalities found in bean seedlings were the result of rough treatment of the seed during threshing, attempts were made to produce the same effects artificially. For this work samples of hand-threshed beans were subjected to various types of mechanical injury. The beans of one lot were placed flat on the table and pressed until they could be heard to crack. In most cases no external injury to the seed coat was evident as a result of this treatment. The beans of another lot were placed on end in a small depression in a board, with the hilum directed to the front and the micropyle above. Each bean was then hit a sharp blow directly over the radicle and hypocotyl by the release of a steel spring. The beans were caught on cloth to prevent a second shock. Slight external injury resulted from this treatment. The beans showing injury to the seed coat were planted separately from the others.

These two lots of injured beans were germinated along with a lot of uninjured hand-shelled material. The uninjured seed produced normal seedlings except for a few cases of baldhead and loss of cotyledons. The seeds which had been subjected to pressure produced seedlings with a very high percentage of cracked and nussing cotyledons, but practically no seedlings with injured hypocotyls or radicles. In the beans which were hit with the steel spring, there was a high percentage of injury to the hypocotyl or radicle. No significant difference appeared in this lot, however, between beans with external injury and those without. A few showed injuries to the hypocotyl similar to those of Figure 5, although in most cases the injury was so severe that the radicle and sometimes a part of the hypocotyl also were completely lost. In this lot of seedlings over 40 per cent showed some type of injury to the hypocotyl or radicle similar to injuries found in machine-threshed material. This observation seems to prove rather definitely that machine threshing is responsible for much of the injury found in bean seedlings.

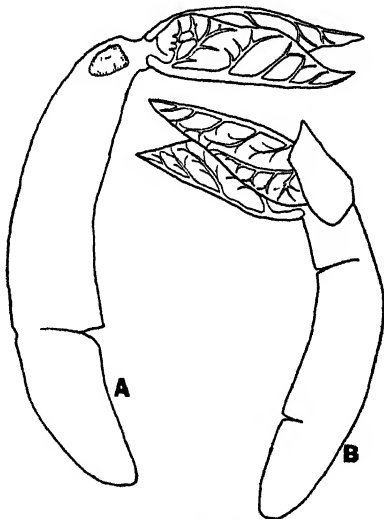


FIGURE 5.—Embryos dissected from soaked seeds. Cotyledons removed to expose injuries to hypocotyl. The embryo at A would probably give rise to a seedling similar to that of Figure 3, D, and the one at B, to one similar to that of Figure 4, D.

#### SUMMARY

Baby Lima beans are found to be very susceptible to thresher injury. In addition to baldhead, which has been described by Harter and shown by him to be largely the result of thresher injury, the following additional types are here described and illustrated. (1) One or both cotyledons may be broken from the embryo; or, in other cases

## AMOUNT OF INJURY IN MACHINE-THRESHED BEANS

The relative amount of seed which produces injured seedlings of the types described in the preceding pages varies markedly in baby Lima beans from different sources. Twenty different lots of beans were obtained from growers near Sacramento, Calif., for germination studies. This material was germinated in the greenhouse in flats containing a rather coarse soil to insure good drainage. Observations were made at the end of about two weeks, at which time the primary leaves had nearly reached their full size and the cotyledons had not yet been entirely depleted of food reserves. Results obtained with these beans are shown in Table 1.

TABLE 1—Summary of germination results from 20 lots of baby Lima beans germinated in soil flats in the greenhouse

[Figures represent percentages based on the average of three lots of 40 beans each]

Description of seedling...	Percentage germination of beans in lot No —																				Average germination
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Normal <sup>a</sup> .....	63	75	65	57	55	43	85	55	70	76	63	53	59	71	54	25	84	72	77	94	64.8
Jointed hypocotyl <sup>b</sup> .....	8	5	14	19	21	28	5	7	7	10	15	12	14	14	9	26	2	0	1	1	10.9
Baldhead <sup>c</sup> .....	7	5	3	7	7	4	0	11	8	4	5	10	9	7	8	12	4	1	2	2	5.8
One cotyledon missing.....	7	2	7	6	5	11	4	8	8	2	5	12	7	4	8	5	2	14	11	2	6.5
Two cotyledons missing.....	2	2	3	1	2	0	0	2	0	0	0	2	0	1	1	1	2	0	0	0	1.0
Total seedlings.....	87	89	92	90	90	86	94	83	93	92	88	89	89	97	80	69	94	87	91	99	89.0
Ungerminated seed.....	13	11	8	10	10	14	6	17	7	8	12	11	11	3	20	31	6	13	9	1	11.1

<sup>a</sup> By a "normal seedling" is meant 1 with 2 cotyledons, 2 well-developed vegetative leaves, and an axis with no evidence of injury.

<sup>b</sup> "Jointed hypocotyl" refers to those seedlings in which the hypocotyl has been cracked but the wound has been healed.

<sup>c</sup> "Baldhead" refers to seedlings with the plumule missing.

In considering the amount of injury shown in these 20 lots of seed, one notes that, while baldhead is rather common, other types of injury occur much more frequently. In many cases these other injuries probably do not cause so much reduction in yield as does baldhead. In other cases, however, the injuries are so severe that the plants fail to survive. Many of the ungerminated seeds, it was found, failed to germinate because they were too completely shattered internally to make any growth, although there was no external evidence of this injury at the time the beans were planted. This was found repeatedly to be the case in seeds germinated on blotters, where they could be observed during the early germination stages. Accurate data concerning injury to underground parts of the seedlings grown in soil flats were not secured, because the seedlings were pulled out for inspection instead of being washed out of the soil. Had these injuries been accurately recorded, the number of seedlings showing defects would have been even greater than the table shows.

## ARTIFICIALLY INDUCED INJURY

Hand-shelled beans do not produce seedlings showing the defects described above. A supply of hand-shelled beans was obtained from the field that produced lot 16 of Table 1. Lot 16 was machine threshed, and of the 20 lots investigated it produced the greatest number of defective seedlings. Several hundred of these hand-shelled

# EFFECTS OF NUTRITION AND HEREDITY UPON LITTER SIZE IN SWINE AND RATS<sup>1</sup>

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## INTRODUCTION

It is a common practice with the laymen working with multiparous animals to select breeding stock, both male and female, but more particularly female, from large litters in the belief that the factors governing size of litter are inherited by the progeny from their parents. It is of considerable economic importance to know whether or not litter size can be increased by the selection of breeding stock from the larger litters. If there are environmental factors which affect the size of litter, such, for example, as nutrition, then by improving these factors the breeder should be rewarded by an increase in the productiveness of his animals.

## REVIEW OF LITERATURE

Rommel (17)<sup>4</sup> found the average litter size of Poland China sows for the period 1882-1886 to be 7.04 pigs and for the period 1898-1902 to be 7.52 pigs, an increase of 0.48 pig per litter. Rommel and Phillips (18) observed that the average litter size of 5-year-old Poland China sows was 8.40 pigs, while the average litter size of yearling sows was only 6.65 pigs. King (11) found that very young and very old female rats produced smaller litters as a rule than females of intermediate age. She concluded that both age and physical condition are important factors in the determination of litter size. Johansson (9) and Keith (10) in studies with swine, and Green (5) in studies with mice, among others, have also observed increase in litter size with increase in age of dam.

Wentworth and Aubel (23) found no difference in the average litter size of "big-type" and "small-type" Poland China swine. Their figures were  $7.85 \pm 0.05$  and  $7.89 \pm 0.04$ , respectively. No data were given to show the actual difference in the size of the two types.

Hammond (7) concluded that the lower fertility of young sows is to a large extent due to the smaller number of ova shed, since he found the average number of corpora lutea in eight young sows to be  $14.3 \pm 0.39$  and in nine old sows  $19.77 \pm 1.26$ . Loeb (12) found that when guinea pigs were underfed until they had lost up to 35 per cent of their body weight the Graafian follicles failed to develop or developed only partly, resulting in failure to ovulate. The underfeeding produced temporary sterility. He did not, however, report the

<sup>1</sup> Received for publication July 1, 1931; issued May, 1932. Paper No. 1034, Journal Series, Minnesota Agricultural Experiment Station.

<sup>2</sup> National Research Council Fellow.

<sup>3</sup> The authors wish to express their sincere appreciation to Dr. Leroy S. Palmer and Dr. Cornelia Kennedy for permission to use data obtained by them on litter size in rats, and to O. E. Mydland for help in keeping and tabulating the records of litter size in the small-animal breeding colony of the nutrition laboratory of the Division of Agricultural Biochemistry.

<sup>4</sup> Reference is made by number (italic) to Literature cited, p. 520.

where they still adhere to the plant, they may be injured to such an extent that the food reserves are not efficiently translocated (2) Injury to the radicle results in its complete loss. In less severe cases it remains attached but ceases to function. Its place is taken by adventitious roots which arise from the injured areas (3) The hypocotyl may be broken, in which case, if the break is near the top, the cotyledons remain below ground when germination occurs. Frequently the break is incomplete and heals as the seed germinates. This last type of injury does not materially retard the growth of the plant

Injuries of the types described were found to be present in all lots of machine-threshed beans examined and very abundant in some of them. In hand-shelled beans, injuries to the radicle and hypocotyl were completely absent, and injuries to cotyledons and plumule were far less frequent than in machine-threshed beans. Injuries similar to those found in machine-threshed beans were produced in hand-shelled ones by subjecting them to mechanical shock before germination. The conclusion is drawn, therefore, that the defects found in baby Lima bean seedlings are the result of injury received in the threshing machine

produced but 1 litter, which consisted of 4 pigs. Since Wentworth and Lush found the result of their experiment in agreement with that of Simpson, they consider it suggestive of the dominance of the factors for wild litter size. Such results need to be verified by larger numbers, however, before the question is definitely answered.

Harris (8), in an analysis of data presented by Wentworth and Aubel (24), found a statistically significant correlation between the size of the litter in which a boar was farrowed and the size of the litters in which his daughters were farrowed. He also found a correlation of  $+0.121 \pm 0.022$  between the size of the litters in which the grandsires and granddams were farrowed. Both these correlations are as large as the correlation which Wentworth and Aubel found between the size of the sow's litter and the size of the litter in which she was produced. There is no genetic reason for either of the two former correlations, hence one is led to question the source of the data from which the correlations were determined. Harris believed that such correlations might arise (1) through strains of animals of different breeders differing with respect to fertility, (2) through differences in the conditions under which breeders maintained their herds, provided such differences affected litter size, or (3) through actual dishonesty of certain breeders in reporting the size of litters for herd-book publication.

Buchanan Smith (21), from a review of the literature, reached the conclusion that litter size is definitely inherited as a comparatively simple Mendelian dominant, but says that perhaps hereditary factors are not as important in determining litter size as good husbandry and the mothering ability of the sow. His conclusions regarding the inheritance of litter size as a simple Mendelian dominant appears unwarranted in the light of the data at present available.

Evvard (2, 3) and Evvard, Dox, and Guernsey (4) found that nutrition was an important factor in determining the size of pigs farrowed, but they did not obtain significant differences in litter sizes.

#### EXPERIMENTAL MATERIAL

This study was made in an effort to discover (1) what relation, if any, exists between the size of the litter of which the dam formed a part and the size of litter that she produced, and (2) to determine whether size of litter is affected by the nutrition of the dam. Accurate records from a rat colony maintained by the nutrition laboratory of the Division of Agricultural Biochemistry were available to the writers. This colony is kept in a well-lighted room the temperature of which is maintained between 75° and 80° F. throughout the year. The animals are fed a diet of natural foodstuffs designed to produce normal growth and reproduction (15). In addition to the records from these above animals, the authors were given access to data collected by L. S. Palmer and Cornelia Kennedy, from which it was possible to study the effects of a diet low in nutritive value upon the fertility of the dam.<sup>5</sup> Although the records were taken from an experiment planned for another purpose it is believed that they are entirely suitable for this study because of the accuracy with which they were

<sup>5</sup> This diet consisted of 310 parts cereal grains, 533 parts dextrin, 100 parts commercial casein, 50 parts timothy hay, and 0.3 per cent cod-liver oil.  $\text{CaCO}_3$  and  $\text{Ca}_3(\text{PO}_4)_2$  were included in the diet in such proportions so as to give certain degrees of acidity and alkalinity and certain percentages of calcium and phosphorus.

length of time before normal ovulation recurred after normal feeding was resumed. Workers at the Cornell Agricultural Experiment Station (14) report the results of 20 years of selection in poultry. As a result of mating high-producing birds with high-producing birds on the one hand, and low-producing birds with low-producing birds on the other, two lines were developed showing marked differences in egg production.

Rommel and Phillips (18), in a study of litter size in Poland China swine reported a correlation coefficient of  $+0.06 \pm 0.008$  between the size of the litters in which the mothers were farrowed and the size of litters farrowed by daughters of these mothers. The correlation coefficient decreased from  $+0.108 \pm 0.014$  for yearling to  $+0.032 \pm 0.037$  for 5-year-old daughters. Rommel concluded that there was a small but definite tendency for fecundity to be inherited, although its influence tended to be lost with increasing age. Johansson (9) found no significant correlation between two different litters of the same sow, but when he correlated the average of the first four litters with the average of the fifth to eighth litters, he obtained a correlation coefficient of  $+0.468 \pm 0.07$ . He also found a correlation coefficient of  $+0.129 \pm 0.079$  when he correlated the average size of the first four litters of the mother with the average size of the first four litters of their daughters. From an analysis of data covering 35 years obtained at one of the largest pig-breeding stations in Sweden, Johansson concluded that the fertility of the sow is affected by environmental influences during growth and maturity. This explanation was made by Johansson to account for the variability in litter size which he found in his data but for which he was unable to account to an appreciable extent on the basis of heredity. Hanes (6) obtained data on guinea pigs indicating that the environmental factors which influence size of litter are associated. Pearson and Weldon (22), correlating the size of litter of mother and daughter, concluded that in mice there is no evidence that litter size is inherited. Keith (10), working with 935 litters of seven breeds of swine, found no significant correlation in relation to its probable error between the size of one litter and the size of the succeeding litter when each breed was considered separately, but when all seven breeds were combined he obtained a correlation coefficient of  $+0.34 \pm 0.03$  between the first and second litters and of  $+0.367 \pm 0.04$  between the second and third litters. While these correlations appear to be statistically significant, they probably are of slight biological value because the large differences in litter size for the various breeds which were combined increase the length of the correlation surface and therefore increase the correlation coefficient. To be of biological value such comparisons should be confined to litters produced within a single breed unless it is first shown that there is no significant difference between the litter size of the breeds combined. It is possible that such a study as this, carried out under a carefully controlled environment, would give a different result.

Simpson (20) believed, from the results of a cross between a wild Schwarzwald boar and a Tamworth sow, that there was a definite tendency for litter size to be inherited as a dominant character. Wentworth and Lush (25) bred six Tamworth sows to a wild boar, and because the average litter size was 7.67 pigs as compared to 11 for the Tamworth breed, they concluded that the boar influenced the size of litter. Only 1 of the crossbred daughters reproduced, and she

should have produced litters of the same size. There is also a significant difference between the average litter size of Groups 2 and 4. In Group 5, which consisted of litters obtained from  $F_1$  females produced by crossing two unrelated strains of inbred rats, there is a significant increase in litter size, which must have come about through the influence of hybrid vigor.

Although Group 5 contained too few litters to make the difference observed between Groups 1 and 5 conclusive, the results do indicate the possibility of increasing litter size by crossing two inbred strains and following it by selection.

Figure 1 shows the correlation surface for the litter size of the dams (Group 1) when compared with the size of the litters which they pro-

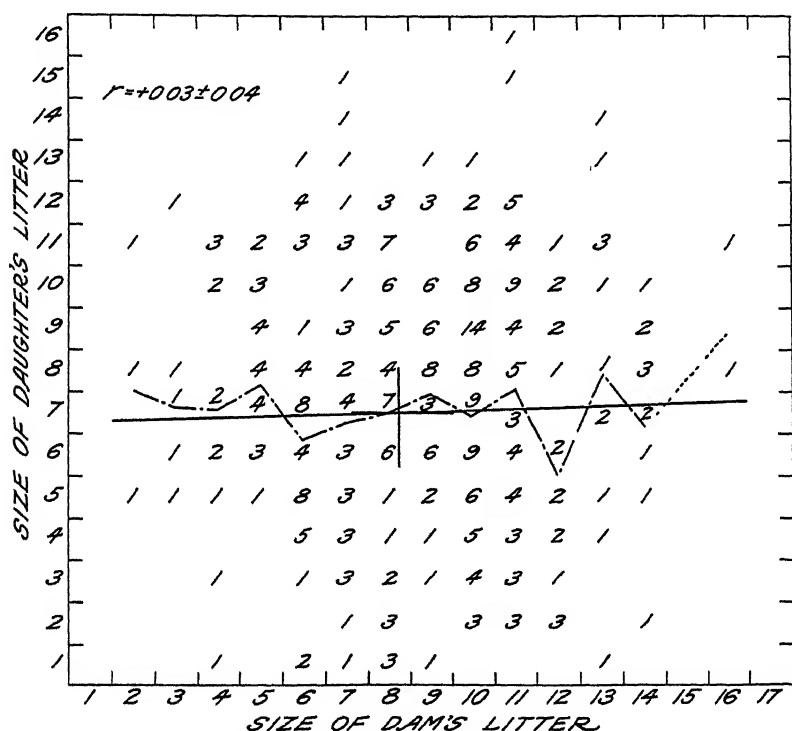


FIGURE 1.—Correlation surface showing relation between size of the litter in which the dam was born and size of the litter she produced, 364 rat litters having been used. The solid line represents the regression line and the dotted line the average size of the daughter litters for each litter size of the dam.

duced (Group 2). The correlation coefficient for this figure is of the same order as its probable error, and is essentially zero. Figure 1 shows the high variability of litter size of the progeny from any class of mothers. It may be concluded from the data here shown that selection of dams from large litters has little influence upon size of litter in rats.

Various workers have found that there is an increase in litter size with the increase in age of the dam. Figure 2 shows a frequency surface for the size of first litter for female rats at various ages. These data give a correlation coefficient of  $+0.10 \pm 0.04$ , an insignificant

kept and because of the inbreeding that has been practiced. In this experiment the females were taken from the normal breeding colony. Most of them were virgin animals. They were placed on a special diet and mated to proven males so that the influence of the sire would not be a factor limiting size of litter.

A study of inheritance of litter size from an economic aspect was also made. Practically all the data for this study were taken from volumes 70 to 81, inclusive, of the herdbooks of the American Poland China Record Association, volume 81 being the last of the herdbooks obtainable at the Minnesota station. Most of the animals used in this study were born between 1918 and 1921. For a discussion of the accuracy of herdbook data the reader is referred to McPhee (13). He found that the herdbook data show fewer litters of 1 to 4 and 9, 11, and 12 pigs than did the experimental data, but the frequency of litters of 8, in the herdbook data, was almost double that in the experimental data. Assuming that all herdbook data are inaccurate and to the extent noted by McPhee, it may yet be said that the inaccuracies affect the litters in which the dams were produced to the same extent that they affect the litters produced by those dams, and probably would not, therefore, affect any correlation that might exist between the size of the dam's litter and the size of the daughter's litter.

#### LITTER SIZE IN RATS

The average size of litters for the various groups of rats was as follows:

	Average number of rats born per litter
Group 1 Litters raised on breeding colony diet; 364 dams from these litters were used in the experiments.....	8.75 ± 0.09
Group 2 Litters produced by these dams (66 per cent first litters).....	7.51 ± 0.10
Group 3 Only first litters from 329 dams whose age at birth of young ranged from 62 to 200 days.....	7.51 ± 0.10
Group 4 One hundred and sixty-two litters produced by dams kept on a diet of low nutritive value and sired by proven males (34 per cent first litters).....	5.67 ± 0.15
Group 5 Litters produced by F <sub>1</sub> hybrids. The hybrids resulted from crossing two unrelated strains (18 litters).....	11.06 ± 0.45

The difference between litter size, in the various groups, where  $E_{diff} = \sqrt{E_1^2 + E_2^2}$  is as follows:

Group 1 minus Group 2 =  $1.24 \pm 0.14$ ; Group 1 minus Group 4 =  $3.08 \pm 0.17$ ; Group 2 minus Group 4 =  $1.84 \pm 0.18$ ; Group 5 minus Group 1 =  $2.31 \pm 0.46$ .

It will be observed that the average size of the dam's litter ( $8.75 \pm 0.09$ ) was significantly greater than the average size of litter produced by these dams ( $7.51 \pm 0.10$ ). This difference is due largely to the fact that a higher percentage of the progeny litters were first litters, as may be seen by comparing the average size of these litters (Group 2) with the average size of first litters (Group 3). The litter size is the same in both groups. If, however, the average litter size in Group 1 is compared with the average litter size in Group 4 (the group on a diet of low nutritive value) a difference of  $3.08 \pm 0.17$  is found—a difference which is highly significant. Thus it is apparent that environment may prevent the genotype from expressing itself, for the females in these two groups were born from the same stock and

found for the earlier period an average size of litter in this breed of 7.52 pigs. This was an increase of 0.48 pig per litter for the period 1898-1902 as compared to the period 1882-1886. The fact that there has been more than twice the increase in fertility in the Poland China breed from 1900 to 1920 than from 1880 to 1900 would indicate that the factors affecting litter size had had a greater influence during the former period than during the latter. This suggests the possibility that the change in type which occurred from 1900 to 1920 may have been a factor contributing to this increase in size of litter. It will be remembered, however, that Wentworth and Aubel did not find any

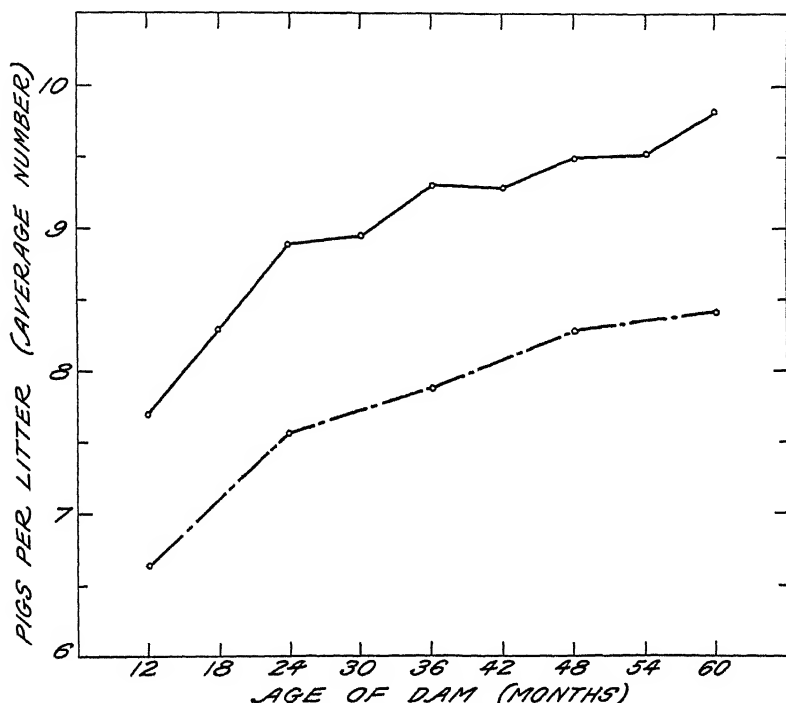


FIGURE 3—Average number of pigs produced per litter by Poland China sows ranging in age from 12 to 60 months. The broken line represents results reported by Rommel and Phillips for the year 1902, the solid line those secured from herdbook records for litters born between 1918 and 1921.

difference in litter size between the large and small type of Poland China swine.

A correlation coefficient showing the relation between the size of the dam's litter and the size of the litter which she would produce was found to be  $+0.11 \pm 0.02$ . There is no significant difference between this correlation and the correlation which Rommel and Phillips found in 1906 in the Poland China breed. From a study of the frequency surface for this correlation as shown in Figure 4, it may be concluded that the selection of dams from large litters would have but a slight effect upon the litter size of their progeny. Since Rommel and Phillips found a greater correlation coefficient for young dams and their progeny than for older dams and their progeny, it was decided that this correlation coefficient should be corrected for the influence of age of dam upon litter size. When this was done by the

value for the numbers studied. This finding is in agreement with that of Johansson (9) in regard to the relation between age of dam and size of first litter in swine. To determine whether the size of the second litter of rats is related to the size of the previous litter, all the second litters, 74 in number, were correlated with the first litters. The resulting correlation coefficient was  $+0.24 \pm 0.07$ . While the correlation is not large and is not based on a large number of litters, it does indicate the possibility of increasing litter size by selection.

#### LITTER SIZE IN THE POLAND CHINA BREED OF SWINE

From the American Poland China Record 1,035 litters were selected at random. Most of these litters, it will be recalled, were born between

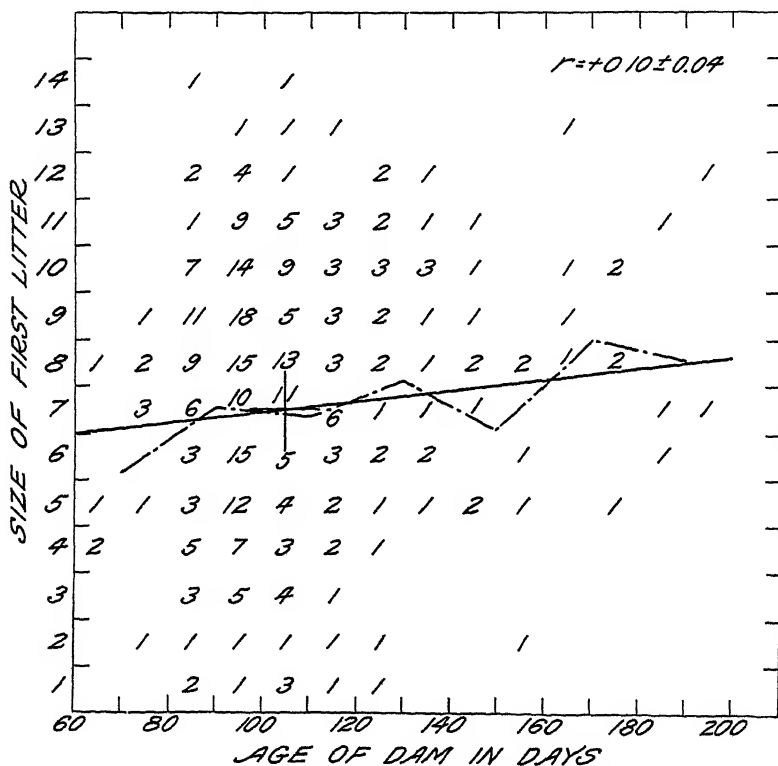


FIGURE 2.—Correlation surface showing relation between age of mother rat in days and size of her first litter. The solid line represents the regression line and the broken line the average size of litter for rats of different ages.

1918 and 1921. The average size of the dam's litter was  $8.69 \pm 0.047$ , whereas the average size of 1,035 litters produced by them was  $8.57 \pm 0.048$ . A comparison of these values with those of Rommel (17) shows an increase of slightly more than 1 pig per litter during the time that elapsed between Rommel's investigations (1898-1902) and those herein reported (1918-1921). The difference in litter size of  $1.05 \pm 0.05$  between these two groups is very significant. This difference is found to exist rather uniformly for all ages of the dam up to 60 months, as shown in Figure 3. It will be remembered that Rommel

## DISCUSSION

The number of animals born to a multiparous mother is influenced to a large extent by the physiological condition of the female before and at the time of oestrus. Haines (6) in studies of guinea pigs found that the major factors controlling litter size operate at conception. He found also that litter size is generally small from January to April, while from June to November it is unusually large. It seems probable that this difference is due largely to the nutrition of the mothers, for it is much easier to procure suitable green feed in summer than in late winter or early spring.

The size of litter depends primarily upon the number of ova which are released and which become fertilized. Parkes and Drummond (16) believe that any male capable of producing viable sperm is capable of fertilizing all of the ova produced. Warwick (23), in an examination of 3,967 fetuses, found 3.68 per cent in various stages of degeneration. It is not known, of course, to what extent heredity and environment, respectively, may account for this. There are many physiological conditions which affect the general health of the animal, and which would result in a smaller litter size. The data presented in this paper show that in the case of rats poor nutrition of the dam is one of the major factors affecting the number of young born per litter. It seems very probable, therefore, that improved feeding methods have influenced the measurable increase in litter size of the Poland China breed during the years 1885 to 1900 and 1900 to 1920.

Differences in litter size of breeds of swine have been definitely established by Bitting (1), Rommel (17), and Severson (19). Severson reported a litter size of 8.2 for Poland Chinas, Keith (10) found 7.91 during the period 1903-1925, Rommel reported 7.52 in 1906, and the present study shows 8.69. Such results as these suggest that there may be differences in litter size within a breed, an idea that is in keeping with the theory advanced by Harris (8) that strains of animals from different breeders may differ with respect to fertility. This difference, if it actually does exist, may not indicate any real difference in the fertility of the strain in question but may be explained upon a nutritional basis, that is, sows maintained at different locations and by different breeders may also be maintained on different planes of nutrition. This idea is supported by the studies of Johansson (9), who found that there had been no change in litter size at Bondeson's pig breeding station in Sweden for 35 years, where undoubtedly the best feeding practices were employed at all times.

If the size of litter is a valuable criterion in the selection of breeding stock, then the number in the litter of which the dam was a part should give an indication as to the size of litter that she will produce, and the size of the first litter should give an indication of the size of subsequent litters. The present study with rats and Johansson's study with swine show that the average size of litter produced by all individuals born in litters of any given size, as 10 for example (figs. 1 and 4), will be the average size of litter for the breed or species studied. The same is true of the size of second litters produced by dams all of which had produced the same size of first litters. This can be accounted for by environmental factors which affect litter size.

partial correlation coefficient method, it was found that  $a^r_{md} = +0.092 \pm 0.02$ <sup>6</sup>. Only a slight decrease in the correlation coefficient was thus obtained when the age of the dam was made constant.

Since in Figure 3 there was a gradual increase in litter size with increase in age of dam up to 60 months, it was thought that there should be a fairly high correlation between age of dam and size of the litter that she would produce. This correlation was found to be  $+0.31 \pm 0.02$ , which is significant. These data, therefore, further

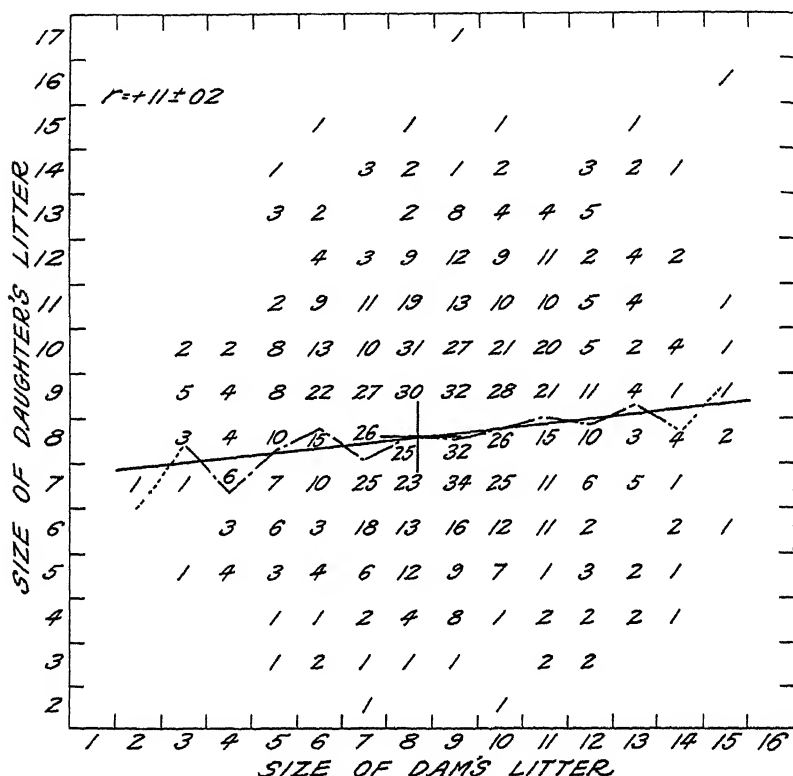


FIGURE 4.—Correlation surface showing relation between size of litter in which the dam was born and size of litter she produced, 1,035 litters of Poland China swine being represented. The heavy line represents the regression line and the dotted line the average size of the daughter litters for each litter size of the dam.

verify the fact previously shown that there is a gradual increase in fertility of sows up to the age of 60 months.

Johansson (9) found a somewhat larger average litter size for sows farrowing their first litter at 14 to 16 months than at any other age up to 22 months, but considering the numbers with which he worked the differences he obtained were not statistically significant. The correlation between age of dam and size of first litter for 262 dams ranging in age from 10 to 15 months, inclusive, was also found in this study to be statistically insignificant. This is in agreement with Keith's (10) findings and also in agreement with the data for rats presented in Figure 2.

<sup>6</sup>  $a$ =age of dam,  $m$ =size of dam's litter,  $d$ =size of daughter's litter  $a^r_{md} = \frac{r_{md} - r_{am} r_{ad}}{\sqrt{1 - r_{am}^2} \sqrt{1 - r_{ad}^2}}$

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It does not, however, disprove the idea that litter size is or may be inherited, for it is well known that breeds differ with respect to litter size

### SUMMARY

A study of 1,035 litters of Poland China pigs, as derived from herd-book records, shows that there was an increase in average litter size amounting to one pig per litter in this breed between 1900 and 1920

The swine data show an increase in average litter size with increase in age of dam up to 60 months

The correlation coefficient between the size of litter in which the dam was born and the size of litter produced by her was very low in swine and essentially zero in 364 litters of rats

The nutrition of the mother rat has a pronounced influence on the size of litter that she produces. Small litters result when the female is maintained on a poor diet

The correlation coefficient obtained between age of dam and size of first litter in both rats and swine was found to be statistically insignificant

The data used in this study show that, because of the influence of environmental factors on litter size, the size of the first litter is not an accurate indication as to what will be the size of subsequent litters. Consequently, while the data indicate the possibility of increasing litter size by selection, the size of the first litter should not be taken as the standard by which to select stock for breeding purposes.

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# CORRELATIONS OF CERTAIN LINT CHARACTERS IN COTTON AND THEIR PRACTICAL APPLICATION<sup>1</sup>

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## INTRODUCTION

Correlation is a tool that the plant breeder has long used in his efforts to breed desirable characters into new varieties. In relatively few instances, however, has correlation been of very great practical importance to plant breeders. In most cases the simple correlation coefficients alone, unless exceedingly high, have in themselves little value except as a means of calculating the partial, or net, and multiple correlations. For correlation coefficients to be reliable it is necessary that there be included in the calculations all measurable factors that might exert an influence on the particular character desired. When several characters or variables are used in the calculations it is not uncommon to obtain a high positive or negative simple correlation coefficient that will be entirely reversed when the partial or net correlation coefficient is computed.

This paper reports data on the relationships of percentage of lint, lint index, boll weight, and length of lint in certain related families of cotton. The simple and partial correlation coefficients between each of these characters are given, as are also the multiple correlation coefficients where each of the characters is used as the primary. The practical application of the data to breeding procedure is shown.

## MATERIALS AND METHODS

In the spring of 1929 seed of a hybrid plant raised on the agronomy farm of the New Mexico Agricultural Experiment Station was planted under the number 179. The progeny segregated for practically all the characters of the cotton plant. Probably the hybrid was an upland-Egyptian cross, as it exhibited characters of both types. The families shown in Tables 1 to 3 were grown in 1930 from different individual plants of No. 179.

The plants grown in 1930 were harvested in the late fall after most of the bolls had opened, and the cotton was then ginned on a small 8-saw gin. The percentage of lint was calculated from the weight of clean lint and clean seed. The lint index was calculated from the formula:  $\frac{\text{Weight of 100 seeds} \times \text{per cent lint}}{\text{Per cent seed}}$  (8).<sup>2</sup> Boll weight was calculated as the weight of lint in grams per boll. Length of lint was measured in sixteenths of an inch.

The correlations were calculated by the usual methods. The simple correlation coefficients were determined first and from these the multiple and partial correlations were obtained.

<sup>1</sup> Received for publication July 15, 1931, issued May, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 527.



Griffie, Ligon, and Brannon (3) report simple high correlation coefficients between the above-mentioned characters similar in size and direction to those reported here. Their simple coefficient  $-0.872 \pm 0.037$ , between number of bolls per pound and weight of 100 seeds was reduced in the partial correlation coefficient to  $0.159 \pm 0.151$  when four other characters were held constant. The number of bolls per pound refers to boll weight, and the weight of 100 seeds to lint index, although they are not exactly the same.

Kearney (5), working with Pima cotton, obtained significant positive simple correlations between each pair of the three characters percentage lint and lint index, lint index, and boll weight, and percentage lint and boll weight.

The present writer (10), in a study of 7 varieties of cotton at the New Mexico station, obtained partial coefficients between percentage of lint and boll weight similar to those reported here. In 4 of the varieties fairly significant positive correlations were obtained, in 1 variety the correlation was barely significant ( $0.20 \pm 0.07$ ), in 1 variety it was not significant ( $0.06 \pm 0.07$ ), and in 1 variety it was negative ( $-0.25 \pm 0.07$ ).

Hodson (2) reported simple coefficients for lint percentage and boll weight, 1 negative ( $-0.45 \pm 0.09$ ), 1 positive ( $0.40 \pm 0.06$ ), and 3 that were not significant.

The genetic factors expressed in percentage lint and lint index, and lint index and boll weight seem to be the same, or, if different, they are closely linked. However, there are genetic factors other than these expressing themselves on each character, since the degree of correlation is not high enough to be explained on the basis of complete linkage of all the genes involved.

The relation of percentage of lint and boll weight may be explained on the basis of two linked genes with crossing over, the other genes being independent. Certain of these genotypes would segregate into a repulsion phase, others into the coupling phase, and still others into an independent phase in which only one of the linked genes would be present. In these nine sister families this could easily explain the range of the partial correlation coefficients from 0.62 to 0.40. Still, it seems logical to assume that if A and B<sup>3</sup> are correlated and B and C are correlated, A and C should be correlated. This was the case with the simple coefficients, but when the partial coefficients were obtained only three families were found to exhibit significant positive correlations. Genetically, then, there must be one gene of C linked with one of A that is not accounted for in B, whose true coefficient is covered up by the common association of the others.

#### LENGTH OF LINT AND OTHER CHARACTERS

In regard to length of lint and percentage of lint, most writers (2, 3, 4, 5, 7, 9, 10) have reported significantly negative correlations, but some writers (4, 9, 10) have found also a number of correlations that are not significant. Kottur (6), working with Indian cottons, reported independent inheritance in these two characters. In the nine sister families of the present study the correlation coefficients ranged from  $-0.61$  to  $-0.01$ . The high negative correlation may be explained on the basis of two factors closely linked. One of these affects percentage of lint and the other length of lint. The coefficients showing no correlation may be explained as the expression of certain factors where not more than one of the linked genes is heterozygous.

## THE DATA

The mean, standard deviation, and range are given in Table 1 for each of the characters for all the families. The simple and partial correlation coefficients between the four characters for each family are shown in Table 2, and the multiple correlation coefficients in Table 3

## LINT PERCENTAGE, LINT INDEX, AND BOLL WEIGHT

An examination of the partial coefficients shows that percentage of lint and lint index, as well as lint index and weight per boll, are significantly positively correlated, but the coefficients for percentage of lint and boll weight range from fairly positive to fairly negative.

TABLE 1—Range, mean, and standard deviation of percentage lint, lint index, boll weight, and length of lint of nine sister families of cotton

Family No	Number of plants	Percentage lint			Lint index		
		Range	Mean	Standard deviation	Range	Mean	Standard deviation
33.....	115	14-41	31.5±0.3	4.9±0.2	1.5-9.5	6.15±0.12	1.84±0.08
36.....	68	14-41	28.4±.5	5.9±.3	1.5-9.5	3.78±.13	1.64±.09
37.....	53	20-38	29.6±.4	4.2±.3	2.5-8.5	4.67±.15	1.60±.10
46.....	60	16-41	29.6±.5	5.2±.3	1.5-8.5	4.60±.15	1.77±.11
47.....	50	12-40	28.2±.6	6.1±.4	1.5-8.5	4.46±.19	2.02±.14
53.....	78	3-39	27.8±.6	8.5±.5	5-9.5	5.08±.16	2.05±.11
60.....	85	23-39	31.4±.2	3.4±.2	3.5-9.5	5.77±.09	1.18±.06
69.....	77	12-42	33.4±.3	4.2±.2	2.5-9.5	5.95±.11	1.37±.07
94.....	127	6-42	30.4±.4	6.6±.3	5-9.5	5.05±.12	1.93±.08

Family No	Boll weight			Lint length		
	Range	Mean	Standard deviation	Range	Mean	Standard deviation
33.....	0-3.0	1.60±0.05	0.72±0.03	16-26	19.9±0.09	1.41±0.06
36.....	0-2.5	.82±.04	.51±.03	13-26	18.5±.2	2.7±.2
37.....	0-2.5	.92±.05	.54±.04	15-26	21.4±.2	2.5±.2
46.....	0-2.5	1.13±.05	.62±.04	15-24	19.1±.2	2.0±.1
47.....	0-2.5	.93±.6	.63±.04	16-23	18.8±.2	1.8±.1
53.....	0-3.0	1.33±.06	.73±.04	14-23	19.2±.1	1.7±.1
60.....	5-3.5	1.19±.04	.51±.03	15-22	19.1±.1	1.6±.1
69.....	5-3.0	1.48±.04	.52±.03	15-24	18.8±.1	1.7±.1
94.....	0-4.0	1.22±.04	.68±.03	14-27	20.8±.1	2.4±.1

TABLE 2—Simple and partial correlation coefficients between percentage lint, lint index, boll weight, and lint length in a series of related families of cotton

Family No	Number of plants	AB CD *		AC BD *		AD BC *		BC AD *		BD AC *		CD AB *	
		Simple	Net	Simple	Net	Simple	Net	Simple	Net	Simple	Net	Simple	Net
33.....	115	+0.76	+0.48	0.77	0.41	-0.37	-0.61	0.83	0.47	0.03	0.36	-0.04	0.17
36.....	68	.79	.63	.61	.20	-.45	-.51	.71	.41	-.19	.20	-.09	.16
37.....	53	.61	.46	.48	-.03	-.29	-.30	.76	.69	-.09	.19	-.17	-.15
46.....	60	.86	.55	.79	.24	-.11	-.01	.83	.56	-.03	.38	-.33	-.52
47.....	50	.80	.58	.67	-.04	.14	-.03	.86	.72	-.21	.06	.21	.06
53.....	78	.91	.64	.85	.12	-.31	-.06	.90	.61	-.32	-.07	-.28	.01
60.....	85	.74	.72	.47	-.40	-.07	-.07	.84	.83	.03	-.04	.12	.13
69.....	77	.72	.30	.81	.62	-.16	-.38	.76	.38	.06	.13	.07	.26
94.....	127	.86	.76	.56	-.18	-.39	-.17	.74	.62	-.33	-.13	-.09	.21

\* A, per cent lint, B, lint index, C, weight per boll, D, length of lint

and testing, which will require only fairly large numbers in the breeding program

With respect to the three characters—percentage of lint, lint index, and boll weight—high simple correlations are obtained between each pair, so that in the families in which fairly high partial correlations are found between each pair of the three characters, a breeder using moderately large numbers would be justified in considering only one of the three, for by selecting for one he would automatically obtain, to a certain extent, the others as well

#### SUMMARY

Data on nine sister families showing the correlation relationship between lint percentage, lint index, boll weight, and length of lint are reported.

The families studied sprang from a hybrid plant, which was probably an upland-Egyptian cross

The mean, standard deviation and range are given for all four characters for each of the nine families

Simple and partial correlation coefficients are shown between all characters for each family, and multiple correlations, using each character as the primary, are given for all families

These data are discussed both from the standpoint of genetics and from that of the practical breeder. Lint percentage and length of lint are considered especially in relation to the other characters, and their practical importance is pointed out.

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The simple correlations of B and D are similar to the partial correlations of A and C, although the significant correlations are not so high. These simple correlations agree with those obtained by Kearney (5) on three populations of Egyptian cotton, where the coefficients were  $0.360 \pm 0.055$ ,  $-0.132 \pm 0.038$ , and  $-0.361 \pm 0.085$ . However, the partials of B and D reported here show only two families with significant correlations and in no family was there a significant negative correlation. The genetic relationships of these two characters must be similar to those indicated for A and D, except that negative correlations between A and D were found, whereas the significant correlations between B and D were positive.

While the relation of C and D is similar to that of A and C, shown above, the one significant positive correlation is not very high, being only  $0.26 \pm 0.07$ .

#### MULTIPLE CORRELATIONS

The multiple correlations for the nine families are shown in Table 3. These coefficients vary greatly according to the family, as might be expected in progenies produced by different genotypes.

TABLE 3—Multiple correlation coefficients of percentage lint, lint index, weight per boll, and lint length when each is used as the primary

Family No	A BCD	B CDA	C DCA	D CAB	Family No	A BCD	B CDA	C DCA	D CAB
33-----	0.88	0.87	0.86	0.62	53-----	0.91	0.95	0.91	0.82
35-----	85	81	72	61	60-----	80	93	87	19
37-----	65	82	77	34	69-----	83	78	86	39
46-----	87	91	89	54	94-----	87	92	77	45
47-----	80	91	86	22					

#### PRACTICAL APPLICATION OF DATA

In practical breeding procedure the partial correlation coefficients shown above are of some importance. Especially is this true of the relationship between lint percentage and length of lint. Cotton breeders have long recognized the negative correlation existing between percentage of lint and length of lint. Both of these characters are important to the commercial breeder because of the farmers' demand for a high percentage of lint and a good length of staple. The negative correlations obtained by most investigators have discouraged some breeders. Of course, if a breeder were selecting within a relatively pure strain for these particular characters and there was a negative correlation, it would be doubtful whether he could obtain both high percentage of lint and good length of staple, even with exceedingly large numbers. On the other hand, should there be no correlation, the maximum possibilities could easily be attained.

In regard to the relationship of percentage lint with boll weight, which certainly shows genetic correlations (1), three courses would be open to the breeder. In the case of the families showing positive correlations he would merely take for further testing those individuals with high percentage lint and high boll weight and the chances would be good that some would prove to be pure the following year for the characters desired. This would also be true for the families showing negative correlations, except that the individuals high in both characters would probably not be very numerous, and therefore a longer time would be necessary to obtain the desired characters. In the case of the noncorrelation families the matter is one of individual selection.

# A SIMPLE METHOD OF CONSTRUCTING TREE VOLUME TABLES<sup>1</sup>

By D. B. DEMERRITT, *Associate Professor of Forestry*, and A. C. MCINTYRE, *Instructor in Forest Research, Pennsylvania Agricultural Experiment Station*

## INTRODUCTION

A volume table shows for a given species the average contents of trees of different sizes (5).<sup>2</sup> In the past, most volume tables have been constructed by separating the field data or samples into diameter and height classes, plotting the class averages, drawing smooth curves and harmonizing them with each other.

Recently Reineke and Bruce<sup>3</sup> have referred tree volume to that of modified cylinders or frustums of ideal solids in the construction of alinement chart volume tables. Three factors, diameter, height, and form of tree, affect tree volume. Within any diameter-height group variations in form produce corresponding variations in volume. These three factors are harmonized through averaging, and the resulting table presents the assumed average trees for the universe from which the sample was taken.

If the sample is a true average for the whole and the mathematical and mechanical work of computation and curve construction have been carefully done, the other parts of the unit from which the sample came can be measured correctly by using the resulting tabular tree volume table or alinement chart volume table.

Bruce (2), Bruce and Reineke (3), and Reineke (7) have shown how alinement charts may be employed in the solution of other problems in forest mensuration. The development of the technic outlined in this paper was suggested by their articles.

Alinement charts have certain advantages over the older methods of volume table construction. Because of the fact that all of the data are used in the construction of a single curve, better curve definition results and fewer data are necessary. When data are deficient in a few diameter or height classes, the projection of the curve through these points, connecting with points having sufficient weight, is possible. It should be remembered, however, that usually the ends of the curves are defined by the smallest number of samples and extensions are open to error. Less time is required by the alinement chart method.

## PROCEDURE

A knowledge of the several types of graph paper is assumed. In the preparation of alinement charts involving multiplication or division, some form of logarithmic paper should be used. The scales on such paper simplify the mechanical work of graduating the axes and make logarithmic computations unnecessary.

The present technic of volume table construction is based upon the use of log-log paper, since tree volume computation involves a multiplication. The equation of this multiplication is  $y = ax^2$  ( $h f$ ),

<sup>1</sup> Received for publication July 27, 1931, issued May, 1932.

<sup>2</sup> Reference is made by numbers (*italic*) to Literature Cited, p. 539.

<sup>3</sup> REINEKE, L. H., and BRUCE, D. THE PREPARATION OF ALINEMENT CHART VOLUME TABLES. 1928 [Unpublished manuscript, Forest Service].



TABLE 1—*Classification of trees by d b h regardless of height*—Continued

D b h class (inches)	Trees	Total d b h	Total height	Actual tree volume	Form factor	Tabular volume
	<i>Number</i>	<i>Inches</i>	<i>Feet</i>	<i>Cu ft</i>		<i>Cu ft.</i>
7.....	1	6 7	39 5	3 37	-----	4 04
	5	34 5	226 0	20 42	-----	23 52
	16	113 0	883 4	96 23	-----	99.17
	5	36 5	325 2	37 25	-----	40 12
	Total.....	27	190 7	1,474 1	157 27	166 85
	Average.....	7 06	54 60	5 82	0 393	6 18
8.....	1	7 6	37 5	4 42	-----	4 95
	3	25 2	133 6	23 56	-----	20 98
	17	135 1	940 6	133 74	-----	134 22
	3	24 9	188 6	28 39	-----	29 85
	Total.....	24	192 8	1,300 3	190 11	190 00
	Average.....	8 03	54 18	7 92	.415	7 92
9.....	3	27 6	146 4	30 25	-----	27 55
	5	45 5	282 5	53 31	-----	52 40
	6	55 3	374 8	71 03	-----	71 60
	Total.....	14	128 4	803 7	154 59	151 55
	Average.....	9 17	57 41	11 04	.419	10 83
10.....	1	10 4	45 5	12 66	-----	10 70
	13	131 5	742 0	170 64	-----	166 20
	3	30 4	225 4	50 21	-----	52 30
	12	120 3	746 6	162 31	-----	164 90
	Total.....	29	292 6	1,759 5	395 82	394 10
	Average.....	10 09	60 67	13 65	.405	13 59
11.....	1	11 5	45 5	11 54	-----	12 85
	3	32 8	169 7	43 97	-----	43 15
	11	122 5	711 5	192 57	-----	191 30
	2	21 5	143 8	32 18	-----	36 95
	Total.....	17	188 3	1,070 5	250 26	284 15
	Average.....	11 08	62 97	16 48	.391	16 72
12.....	1	11 7	57 5	16 49	-----	16 60
	7	83 5	455 0	137 09	-----	138 50
	1	11 9	71 0	22 87	-----	22 00
	Total.....	9	107 1	583 5	176 45	177 10
	Average.....	11 90	64 83	19 60	.392	19 18
13.....	1	12 6	52 0	16 19	-----	17 50
	3	39 0	198 5	81 10	-----	73 70
	4	51 9	302 1	116 14	-----	113 40
	3	38 6	247 9	104 15	-----	90 10
	Total.....	11	142 1	800 5	317 58	294 70
	Average.....	12 92	72 77	28 87	.436	26 79
14.....	3	41 8	200 9	76 16	-----	90 30
	4	56 3	293 5	139 45	-----	137 20
	5	70 1	412 7	182 99	-----	186 10
	Total.....	12	168 2	907 1	393 60	413 60
	Average.....	14 02	75 60	33 22	.411	34 46
15.....	1	15 0	58 5	33 10	-----	30 40
	1	14 6	68 8	39 45	-----	34 30
	7	104 7	532 8	285 61	-----	287 10
	1	15 0	85 0	48 16	-----	45 00
	Total.....	10	149 3	745 1	406 32	396 60
	Average.....	14 93	74 51	40 63	.449	39 68
16.....	1	16 3	76 0	52 91	-----	50 50
	2	32 2	162 5	115 19	-----	103 50
	Total.....	3	48 5	238 5	168 10	154 30
	Average.....	16 17	79 5	56 03	.616	51 43
17.....	1	17 2	63 5	45 67	-----	47 00
	4	67 8	291 8	194 02	-----	211 40
	Total.....	5	85 0	355 3	239 69	258 40
	Average.....	17 0	71 06	47 94	.428	51 68
18.....	1	18 0	68 5	50 39	-----	56 60
	1	17 9	89 0	77 67	-----	69 00
	Total.....	2	35 9	157 5	128 06	125 60
	Average.....	17 95	78 75	64 03	.463	62 80
Grand total.....	209	1,968 0	12,262 2	3,139 73	-----	3,135 42

in which  $y$  equals volume,  $a$  is a constant,  $x$  is the diameter, 2 is the exponent of  $x$ ,  $h$  is height, and  $f$  is form factor (5). The substitution of different values of  $x$  in this equation, allowing height and form to remain constant, produces a parabolic curve. A parabolic curve on log-log paper plots as a straight line.

To simplify the explanation of the technic presented, there are included in this paper the average values from a sample of 209 red oak (*Quercus borealis maxima* M.) trees secured on five logging operations in central Pennsylvania. The data were collected in the usual manner (1), and the tree volumes were computed by cubing the logs according to Smalian's parabolic frustum formula. The d b h (diameter at breast height measured at 4.5 feet above ground level) taken outside bark, is an average to the nearest tenth inch of two measurements taken at right angles to each other with tree calipers. Heights, in feet, were measured from the ground level to the tip of the main stem. The volumes shown are total volumes inside bark contained in the stem and limbs, in cubic feet. Volumes do not include stump. Utilization is to a 2-inch top inside bark.

The data, charts, and tables presented are used only for purposes of illustration. Additional samples for this species should be obtained if final charts and tables are to be constructed.

#### ARRANGING THE DATA

The trees are first classified and listed by d b h height classes. Column totals for each class are then obtained. This method necessitates only one listing of the data and reduces computing time by nearly one-half. The units of classification are entirely arbitrary. In this case the d b h classes used are 3.5 to 4.4 inches, 4.5 to 5.4 inches, etc., and the height classes are 30 to 39.9 feet, 40 to 49.9 feet, etc.

The data are then classified by d b h classes regardless of height (Table 1, columns 3, 4, and 5). The totals found in the first classification are used, and the number of trees involved in each class are noted. The number of trees, d b h, height, and volume for each d b h class are totaled and averaged.

In the same way, the trees are classified by height classes regardless of d b h (Table 2, columns 2, 3, and 4) and the average total height and volume in each height class computed. The totals computed in the first classification are again used.

TABLE 1—Classification of trees by d b h regardless of height

D b h class (inches)	Trees	Total d b h	Total height	Actual tree volume	Form factor	Tabular volume
	<i>Number</i>	<i>Inches</i>	<i>Feet</i>	<i>Cu ft</i>		<i>Cu ft</i>
4.....	6	26 2	220 9	9 83	-----	9 89
	4	16 9	180 5	7 42	-----	7 41
	1	4 3	55 0	2 43	-----	2 32
	Total.....	11	47 4	456 4	-----	19 62
Average.....		4 31	41 49	1 79	0 427	1 78
5.....	3	14 0	107 0	5 34	-----	5 45
	3	15 9	187 5	8 98	-----	9 59
	14	72 1	626 1	36 31	-----	36 60
	Total.....	20	102 0	890 6	-----	51 64
Average.....		5 10	44 53	2 53	400	2 58
6.....	2	11 8	75 6	4 93	-----	5 90
	9	53 0	430 5	32 69	-----	32 78
	4	24 9	213 5	18 95	-----	18 23
	Total.....	15	89 7	719 6	-----	56 91
Average.....		5 98	47 97	3 77	404	3 79

# GRADUATING THE DIAMETER AXIS

On log-log cross-section paper, with the abscissa as d. b. h. and the ordinate as volume, the average values obtained in the second classification (Table 1) are plotted. A curve is fitted to the plotted values after proper weights have been assigned, as shown in the left-hand curve of Figure 1. It may be shown that the variation in the points from a smooth curve is due to differences in the height and form factor of the average tree. Column 6 in Table 1 shows the cylinder form factor of the average tree in each class. Figure 2 shows the

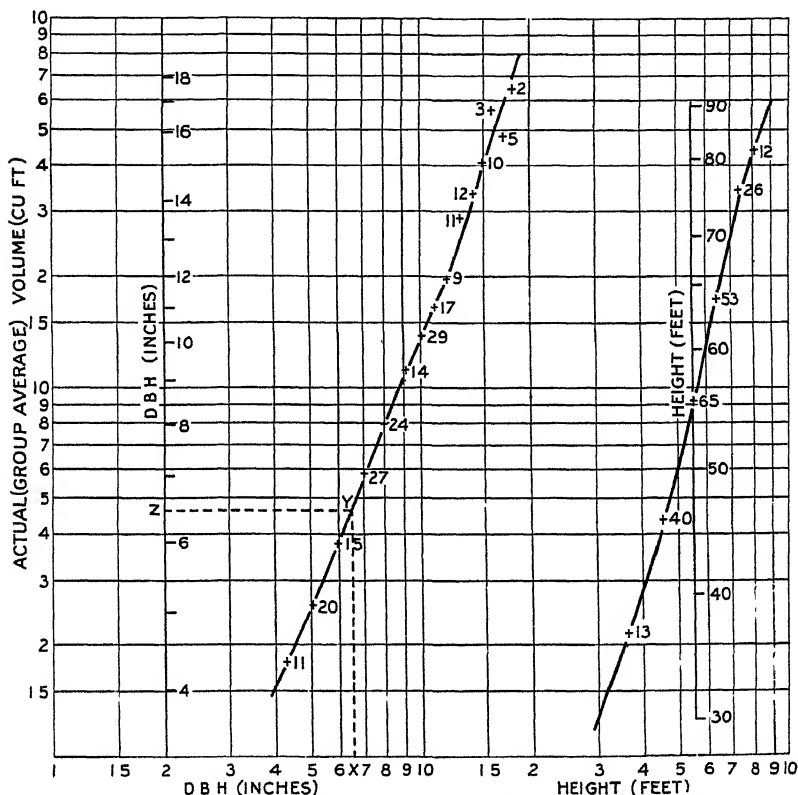


FIGURE 1—Curves showing method of establishing diameter and height axes

extent of these variations and their relation to diameter. It is readily seen that slight changes in form and average height cause the plotted points to deviate from a smooth curve. The diameter classes above 12 inches show more radical changes in form factor, hence greater deviation from the trend of the other plotted values. In cases where more data are available the curve tends to become smoother owing to better sampling.

By using any convenient point near the left of the paper (in this case at abscissa value 2) the d. b. h. axis is established. If, now, the d. b. h. volume curve is used as a graduating curve, the graduations for d. b. h. may be placed on this axis. Even inches only are grad-

TABLE 2.—Classification of trees by height regardless of *d b h*

Height class (feet)	Trees	Total height	Actual tree volume	Tabular volume
	Number	Feet	Cu ft	Cu ft
30.....	6	220 9	9 83	9 89
	3	107 0	5 31	5 45
	2	75 6	4 03	5 90
	1	39 5	3 37	4 04
	1	37 5	4 42	1 95
Total.....	13	480 5	27 89	30 23
Average.....		36 96	2 14	2 32
40.....	4	180 5	7 42	7 41
	14	626 1	36 31	36 60
	9	430 5	32 69	32 78
	5	226 0	20 42	23 52
	3	133 6	23 56	20 98
	3	146 4	30 25	27 55
	1	45 5	12 66	10 70
	1	45 5	11 54	12 85
Total.....	40	1,834 1	174 85	172 39
Average.....		45 85	4 37	4 31
50.....	1	55 0	2 43	2 32
	3	157 5	8 98	9 69
	4	213 5	18 95	18 23
	16	883 4	96 23	99 17
	17	940 6	133 74	134 22
	5	282 5	53 31	52 40
	13	742 0	170 64	166 20
	3	169 7	43 97	43 15
	1	57 5	16 49	16 60
	1	52 0	16 19	17 50
	1	58 5	33 10	30 40
Total.....	65	3,612 2	594 03	589 88
Average.....		55 57	9 14	9 08
60.....	5	325 2	37 25	40 12
	3	188 6	28 39	29 85
	6	374 8	71 03	71 60
	12	746 6	162 31	164 90
	11	711 5	192 57	191 30
	7	455 0	137 09	138 50
	3	198 5	81 10	73 70
	3	200 9	76 16	90 30
	1	68 8	39 45	31 30
	1	63 5	45 67	47 00
	1	68 5	50 39	56 60
Total.....	53	3,401 9	921 41	938 17
Average.....		64 10	17 38	17 70
70.....	3	225 4	50 21	52 30
	2	143 8	32 18	36 95
	1	71 0	22 87	22 00
	4	302 1	116 14	113 40
	4	293 5	139 45	137 20
	7	532 8	285 61	287 10
	1	76 0	52 91	50 80
	1	291 8	194 02	211 40
Total.....	26	1,936 4	893 39	911 15
Average.....		74 47	34 36	35 04
80.....	3	247 9	104 15	90 10
	5	412 7	182 99	186 10
	1	85 0	48 16	45 00
	2	162 5	115 19	103 50
	1	89 0	77 67	69 00
Total.....	12	997 1	528 16	493 70
Average.....		83 09	44 01	41 14
Grand total.....	209	12,262 2	3,139 73	3,135 52

duce a cylinder having a volume equal to that of the cylinder first assumed is computed. Construction lines between the d b h. and height values of these two cylinders are drawn, and the intersection of the two lines in the location of the volume axis. Repetition of this process for several assumed d b h height values will show the exact position of the axis.

It will be found in this case that the volume axis is parallel to the d b h. and height axes and nearer to the d b h. than to the height axis. Its exact location depends upon the range of the d. b h. and

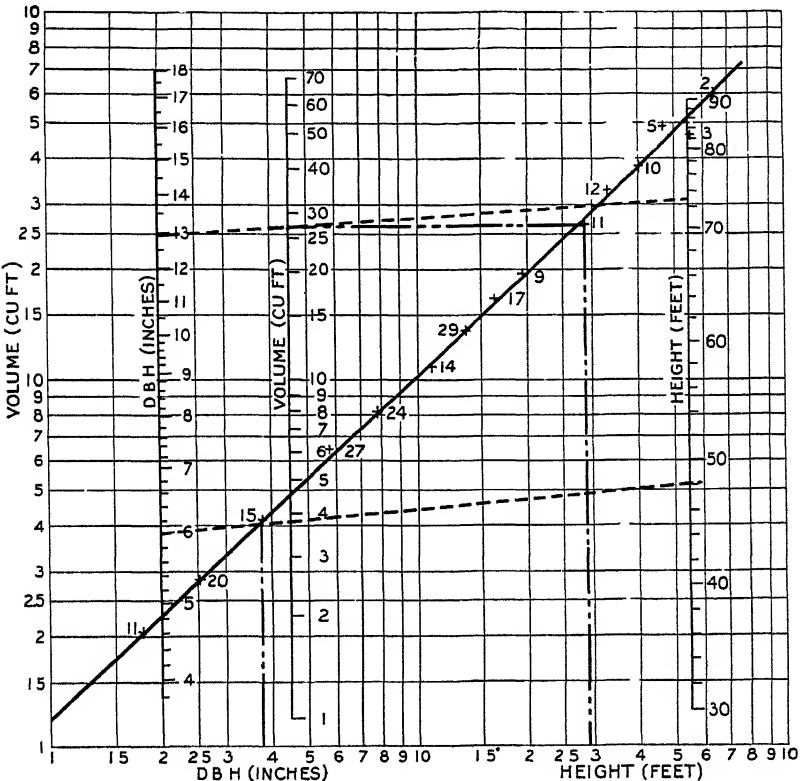


FIGURE 3.—Method of establishing values which locate the volume graduating curve. This drawing shows the form of an alinement chart volume table.

height values and the spacing of their respective axes. A check of the location of volume axis is necessary in constructing a volume table.

GRADUATING THE VOLUME AXIS

Utilizing the average values in Table 1 (Columns 3, 4, and 5), place a straight edge on the d b h. and height values and mark the intersection on the volume axis, as shown by the dotted construction lines for the 6-inch and 13-inch d. b h. classes in Figure 3. Using the abscissa as the actual tree volume, the average volume value is plotted on the abscissa horizontally opposite the intersection obtained by the pairing of the average d. b h.-height values. The double dot and dash construction lines in Figure 3 show the method of locating the

uated in Figure 1, to avoid confusion. The graduations are obtained by tracing vertically from the desired d. b. h. value on the abscissa to the graduating curve intersection, then horizontally to the axis. The dotted line (fig. 1) X-Y-Z shows the method of locating the 6.5-inch graduation.

### GRADUATING THE HEIGHT AXIS

Utilizing the values obtained in the third classification (Table 2), a curve of volume on height is plotted. Volume is the dependent or ordinate value and height is the independent or abscissa value. Se-

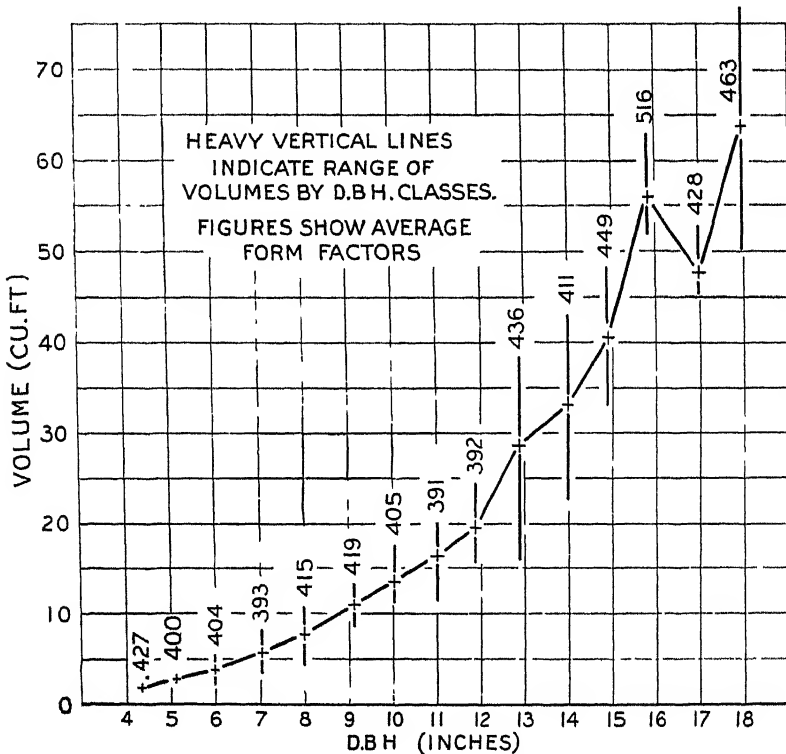


FIGURE 2—Curve showing extent of tree volume variations and average form-factor values for each diameter class

lecting any convenient point near the right side of the graph paper, the height axis is set up. Using the height-volume curve as a graduating curve, the height values are placed on the height axis in the same manner as for the d. b. h. axis. Again, divergence of the plotted values from a smooth curve is due to fluctuations in the form factor of the average tree in the height classes.

### LOCATING THE VOLUME AXIS

To locate the volume axis in cubic volume tables, any convenient d. b. h. and height may be assumed and the volume of a cylinder of these dimensions computed. Another cylinder 2 or 3 inches larger or smaller than the first is then assumed and the height necessary to pro-

For purposes of comparison with the actual tree volumes, the tabular values are totaled by classes. The grand total of the actual tree volumes is compared with the grand total of the tabular volumes. To compute the "aggregate percentage deviation" of the table, the difference between the actual and tabular volumes is obtained. This difference multiplied by 100 is divided by the total volume of the actual trees. A plus or minus sign is assigned to the result depending on the greater volume, tabular or actual.

The usual limit of accuracy for standard volume tables applicable over a large area is 1 per cent. In this particular case the aggregate percentage deviation is  $-0.134$  per cent, which is well within the required limit.

A low aggregate deviation does not necessarily mean that the table as constructed is accurate, since too low volumes in the smaller diameters might offset too high volumes in the larger diameters, or vice versa. A graph of tabular volume (Table 1, column 7) plotted on actual tree volume (column 5) on log-log paper will plot as a straight 45-degree line through 1-1 provided the work is correct. Figure 4 shows a graph in which the plotted values are the averages by d b h classes from Table 1.

Failure of these plotted values to produce a 45-degree line through 1-1 means that the volume axis should be regraduated in those portions as indicated by divergence from the 45-degree line.<sup>4</sup> This is accomplished by reading the ordinate volume value first and then the abscissa value from the curve as the corrected volume graduation. Reading of these corrected volume values at intervals depending upon their magnitude, will allow replotting over the abscissa values on the chart and a new volume graduating curve is produced. The volume axis may now be regraduated and the new individual tree volumes reread from the chart. The graduations on both the diameter and height axis should be carefully checked if considerable variation in tabular values is noted. Recomputation of the aggregate percentage deviation should produce a lower value.

The average percentage deviation is found by determining the percentage deviation of each individual tree volume from its chart volume. The total of these individual deviations taken without regard to sign, multiplied by 100, and divided by the number of trees, gives the average percentage deviation. The limit of this average deviation for standard volume tables should not exceed  $\pm 10$  per cent. The table here produced gives an average percentage deviation of  $\pm 7.74$  per cent.

#### PREPARATION OF THE FINAL TABLE

The volume table may be read from the alinement chart and tabulated in the conventional form (Table 3). Volumes are read for any desired d. b. h. and height interval in the same manner as explained above.

Using the alinement chart itself for determining tree volume makes interpolation unnecessary. For practical application it is, therefore, simpler to use the chart in its finished form rather than in the conventional table form.

<sup>4</sup> REINEKE, L. H., and BRUCE, D. Op. cit.

volume values for the 6-inch and 13-inch classes, and these are respectively weighed with the number of trees in those classes

A smooth curve is fitted to the plotted volume values thus obtained. It will be noted that the divergence from a straight line in these plotted values is less than in the case of the first curve, since d. b. h. is now associated with height, and the latter tends to influence the form factor in the reverse direction

The volume graduations may now be placed on the volume axis, the volume curve being used as a graduating curve. In Figure 3, the major volume values are shown on the volume axis.

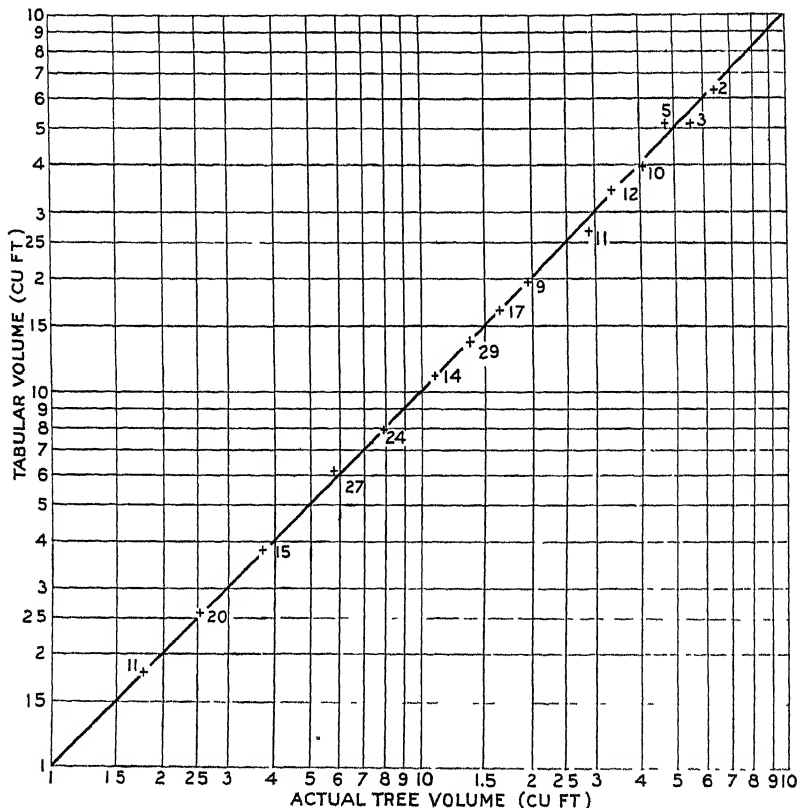


FIGURE 4—Curve showing relation of tabular volume to actual tree volume

#### DETERMINATION OF ACCURACY

In order to check the accuracy of the graphic work (4) and to determine the limits of applicability of the table, it is necessary to read from the completed chart the volume of each individual tree used in its construction.

To determine the volume of any tree, a straight edge is laid on the chart intersecting the d. b. h. and height values on their respective axes. The volume of the tree is read at the point where the straight edge intersects the volume axis. These values are shown as tabular values in column 7 of Table 1.

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## DISCUSSION

Previous alignment chart technic for volume table construction has utilized base charts for standard solid figures, cylindrical, parabolic or cone frustums, depending on the type of table desired. The technic has been based upon correlation of the tree volumes with these base charts. The technic here developed departs from previous practice in that base charts are not utilized.

Previous technic in volume table construction has first correlated the dependent variable, volume, followed by a fitting of the independent variables, *d* *b* *h* and height. The present technic departs from previous practice again in that the independent variables are set up first, then the dependent variable, volume, is correlated with the two independents. Meyer (6) has simultaneously developed the same general principle of correlation but used a base chart with which to correlate first the independent, then the dependent variables.

Comparison of the present technic with previous technic, using identical data, indicates a saving of time in construction of graphs and in reduced correlations. In the comparisons made, the average percentage deviations obtained were in each case reduced by the present technic, the reduction ranging from 0.4 to 1.1 per cent.

TABLE 3—*Merchantable volume<sup>a</sup> of red oak stand Pennsylvania, 1930*

D b h (inches)	Volume (cubic feet) of trees of total height (feet) indicated							Basis number of trees
	30	40	50	60	70	80	90	
4.....	1 24	1 52	1 86					11
5.....	1 81	2 22	2 75					20
6.....	2 60	3 20	3 93					15
7.....	3 60	4 50	5 47	6 60				27
8.....	4 70	5 80	7 07	8 80				24
9.....		7 20	8 95	10 90	13 00			14
10.....		8 95	10 90	13 20	15 60			29
11.....		10 55	12 75	15 40	18 50			17
12.....			15 00	18 10	21 90	25 00		9
13.....			17 90	21 70	26 20	30 00		11
14.....			21 60	26 50	32 00	36 30	39 00	12
15.....			25 80	31 50	38 00	43 00	46 00	10
16.....				37 00	44 40	50 00	54 00	3
17.....				42 70	51 00	58 00	62 00	5
18.....				49 00	58 00	65 50	60 00	2
								209

<sup>a</sup> Volume includes stem and limbs inside bark above a 1-foot stump. Utilization limit is 2 inches inside bark. Heavy line indicates range of basic data. Aggregate percentage deviation Table 0.134 per cent low. Average percentage deviation  $\pm 7.74$  per cent. Data collected in 1930 by A. C. McIntyre and T. A. Liefeld.

## SUMMARY

A technic for the construction of alignment chart volume tables has been developed. Graduating curves for *d* *b* *h* and height are plotted on log-log cross-section paper, and these two independent variables are then correlated with the dependent variable, volume, to produce the finished chart.

Except in the case of meager data, no axis regratuation is necessary since the initial graduations conform strictly to the variations in form factor of the trees measured.

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## BIOLOGY AND HABITS OF THE STRAWBERRY LEAF ROLLER, *ANCYLIS COMPTANA* (FROEL.), IN NEW JERSEY<sup>1</sup>

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### INTRODUCTION

A severe outbreak of the strawberry leaf roller (*Ancylis comptana* Froel.)<sup>3</sup> occurred on a 40-acre strawberry field in the vicinity of Hartford, N. J., in 1920, and although extensive spraying was done the results were negative. The available literature on the strawberry leaf roller at that time furnished very meager information as to its biology and none concerning the feeding habits of the young larvae. The biology of the insect in New Jersey was, therefore, investigated for the purpose of finding a vulnerable stage in the insect's development.

### HISTORICAL REVIEW

Probably the first reported injury by the leaf roller in this country is that mentioned by Riley (8)<sup>4</sup> as occurring in northern Indiana in 1866. Riley observed the insect in 1868 and concluded that it was two brooded and passed the winter in the pupal stage. Observations made in Kentucky in 1890 by Garman (7) indicated that the leaf roller was four brooded and passed the winter in the larval stage, maturing the following spring. Somewhat more complete accounts of the insect and its habits were furnished by Stedman (10) in 1901, by Smith (9) in 1909, and by Webster (11) in 1918. According to Smith, who observed the insect in New Jersey for many years, the eggs are laid on the underside of the leaves and the larvae wander to the upper surface as soon as hatched and, for a day or two, feed openly on the upper surface. Smith also stated that the winter is passed by the leaf roller in the pupal stage. Stedman, reporting from Missouri, and Webster, from Iowa, state that the larvae, as soon as they hatch, spin silken webs under which they feed. Stedman found the young larvae on the upper surface, and Webster found them on both surfaces, but he states that the under surface of the leaves is preferred. No mention of the hibernating habits is made by Stedman, but Webster states that in Iowa the winter is passed by the leaf roller as a mature larva which in early spring pupates without feeding. Other writers have frequently reported the presence and destructiveness of the leaf roller, and recently Dunnam (1) has published the results of a season's observations in Iowa on its life history and control.

Although many conflicting observations regarding the life history of the leaf roller were recorded by the early workers, the more recent reports, especially those of Webster, have been substantiated by the

<sup>1</sup> Received for publication Sept. 25, 1931, issued May, 1932.

<sup>2</sup> At the time these studies were made Dr. Fink was assigned to the Riverton, N. J., laboratory of the Division of Truck Crop Insects.

<sup>3</sup> Order Lepidoptera, family Olethreutidae, subfamily Eucosminae.

<sup>4</sup> Reference is made by number (italic) to Literature Cited, p. 557.



## SYNONYMY

This species was first described by Froelich (6, p. 99) in 1828, as *Tortrix comptana*. Specialists working on this group have since placed it in the genus *Ancylys*. Its synonymy includes the following names.

### *Ancylys comptana* (Froelich)

*Tortrix comptana* Froelich, 1828

*Phoropterix comptana* Duponchel, 1844

*Anchylopera comptana* Wilkinson, 1859

*Grapholitha (Phoropterix) comptana* Heinemann, 1863

*Grapholitha conflexana* Walker, 1863

*Anchylopera fragariae* Walsh and Riley, 1869

*Ancylys comptana* Fernald, 1903



FIGURE 1.—The strawberry leaf roller. A, Eggs on the underside of a strawberry leaf, B, folded leaf opened to show a larva, C, a leaf opened to show the pupa, D, the moth. All  $\times 4$ .

## THE MOTH

### DESCRIPTION

The moths average slightly over 1 cm across the outstretched wings. The general ground color is light to dark reddish brown. The fore wings are reddish brown streaked with darker brown and white lines. When the moth is at rest there can be seen on the base of the folded wings a dark area forming a conspicuous deeper brown patch across the middle of the back. The hind wings are dark gray, and both wings have long fringes. (Fig 1, D) For a more detailed description, furnished by Fernald, the reader is referred to Forbes (5).

writer in his studies of the insect in New Jersey. In the following pages many additional facts regarding the habits and biology of the leaf roller are recorded.

#### DISTRIBUTION AND MEANS OF DISPERSAL

Of European origin, the strawberry leaf roller has been known to exist in this country for more than half a century. From the records available it appears that this species is distributed in the North from the Atlantic coast to the Pacific coast and from Canada southward to Virginia, Kentucky, Kansas, Colorado, and California, which, with the exception of Louisiana and Arkansas, form the southern limit of distribution. It seems to be most destructive in the upper Mississippi Valley and in some of the Atlantic Coast States. There are no records of its occurrence in most of the Southern States.

Local infestations of strawberry fields are often caused by the flight of adults from infested fields. On the other hand, infestations of widely separated localities may be brought about by the importation of infested plants. Plants obtained from an infested region may harbor both eggs and larvae. A careful grower may notice the rolled or folded leaves that harbor the more mature larvae and remove them before setting the plants in the field. It is, however, practically impossible to discover all the very young larvae or eggs that may be on the plants.

#### NATURE OF INJURY

The attack of the strawberry leaf roller is confined entirely to the leaves of the plant. Besides the actual injury to the foliage caused by the feeding of the larvae, the normal life of the plant may be disturbed by the folding or rolling of the leaves. The withering of the leaves results in malnutrition of the exposed fruit, which also withers and shrivels. In severe infestations the foliage of infested strawberry fields looks as though it were scorched or burned, and the fruit becomes deformed and small in size and all tends to ripen at one time.

#### BEDS

The greatest amount of injury to old strawberry beds is apparent during June at the end of the first generation of larvae. The greatest injury to newly set fields is caused by larvae of the second and third generations during the summer. In the fall, owing to the heavy growth in foliage of both old and new strawberry plants, injury is not severe unless accompanied by dry weather.

Growers in localities suffering from depredations by the strawberry leaf roller believe that the losses from reduced yield and poor quality of fruit may amount to as much as 50 per cent of the normal crop.

#### FOOD PLANTS

Fortunately the food plants of the strawberry leaf roller are very limited, and injury in this country is confined mostly to the strawberry, raspberry, and blackberry. However, the writer has found it feeding on clover growing in a strawberry field. Fernald (3, p. 50) mentions the following as European food plants: *Potentilla opaca*, *P. verna*, *P. cinerea*, *Dryas octopetala*, *Poterium sanguisorba*, *Thymus serpyllum*, and *Teucrium*.

Dunnam (1) records the average life of 30 females as 14.73 days and of 25 males as 16.56 days. He does not state, however, the months in which the data were recorded.

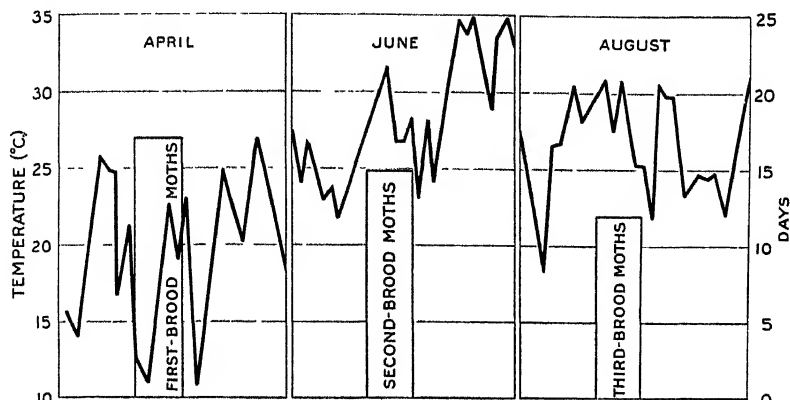


FIGURE 2.—The average length of life of the female moth of the strawberry leaf roller for each of the three broods, and the maximum daily temperatures for the same periods, Riverton, N. J., 1921.

#### PARTHENOGENESIS

To ascertain whether parthenogenesis occurs, unmated and mated females were confined in separate cages with suitable food plants. From 10 unfertilized females thus confined no eggs were recovered, but in cages containing fertilized females kept under similar conditions oviposition occurred. Dunnam (1) states that some unfertilized females in his cage experiments deposited infertile eggs.

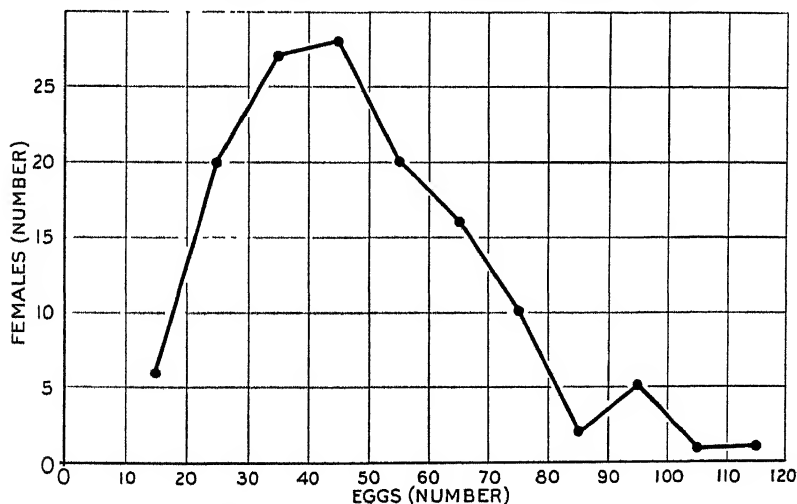


FIGURE 3.—Numbers of eggs deposited by 136 females of the strawberry leaf roller, grouped into classes and each class represented by its mid-point. Riverton, N. J., 1921.

#### FECUNDITY

There is considerable variation in the number of eggs that a moth may deposit. Records were kept of the number of eggs deposited by 136 moths, and these are represented graphically, in Figure 3, in which

## FLIGHT AND PROTECTIVE HABITS

If the moth is disturbed it flies swiftly and erratically from row to row and when it alights on the foliage runs rapidly and secretes itself a short distance from where the flight ended. The moth is probably protected to some extent by the coloration of its fore wings, since the dark-brown spot upon a reddish background blends with the surrounding foliage. When food plants are abundant, the insects do not fly far. Some individuals, however, may find their way to considerable distances and infest new fields. Careful measurements of the distance covered by the moths in a single flight showed a range of from a few yards to 50 feet.

## EMERGENCE, COPULATION, AND OVIPOSITION

The spring adults emerge from the pupae formed from overwintering larvae. The date of emergence is governed largely by weather conditions. In New Jersey, in 1920, moths were observed in strawberry fields the latter part of April, in 1921 they appeared in the fields March 28. In the latter year the month of March was abnormally warm.

Mating was seldom observed in the field. Of several hundred pairs kept in the breeding house for egg records only about six pairs were seen in copula. This number was sufficient, however, to indicate the method and duration of mating. As is the case with many other moths during copulation, the body of the male is extended in line with that of the female and faces in the opposite direction. One pair of moths was observed in copula for over an hour and a half, other pairs for 20 minutes. In the cases observed copulation occurred within a day after emergence, and eggs were deposited three days after mating.

The eggs are normally deposited on the undersides of the strawberry leaves, occasionally upon the upper surface of the leaves, and rarely on the stems. Females confined in cages deposited their eggs on both surfaces of the leaves and sometimes deposited as many as 20 or more upon a single leaf, but this may be attributed to their confinement. In the field usually but one egg is laid on a leaf. Sometimes two or three, but rarely more, are found upon a single leaf. A female may deposit from 20 to 30 eggs during its active oviposition and may repeat the process at intervals of two to three days until all the eggs have been deposited. Oviposition in cages takes place at dusk, and this apparently is true also under field conditions, since at sundown the moths appear to be most active in their flights about the infested fields.

## LONGEVITY

Determinations of the longevity of moths are based upon records of paired individuals used for breeding purposes. The records show that the average life of the male is eight days, but the average life of the female varies somewhat with the season. As shown in Figure 2, the average length of life based on 47 females in April is 17 days; of 34 females in June, 15 days, and 55 females in August, 12 days. Generally it appears that cooler weather is favorable to longevity in this species.

the base of the leaf, or very often along the side of a vein (fig 5). The small larvae gradually construct an overhead covering by spinning threads of silk from side to side. This retreat is at first not much longer than the young larva, but as the feeding area is extended the



FIGURE 5—Feeding areas and the retreats constructed by young larvae of the strawberry leaf roller on the underside of a strawberry leaf.  $\times 2$

tubelike tent is lengthened and broadened to accommodate the insect's growth. Within three or four days the retreat resembles a little funnel, with the narrower end toward the base of the leaf, or if the construction starts along a single vein it may resemble a

it is shown that the greatest number of females deposited from 20 to 70 eggs. The average number of eggs deposited by the moths in different months of the year was found to be as follows: April, 38.6, June, 59.5, August, 52.5. These figures are based on 47, 34, and 55 females, respectively. Webster (11) states that the average number of eggs laid by 35 females was 72.9. Dunnam (1) found that the number of eggs laid per day ranged from 1 to 67 and that the average number laid per female was 85.1.

### THE EGG

#### DESCRIPTION

The egg viewed from above is oval, and has an irregular basal surface which is attached to the leaf. (Fig 1, A.) As light plays upon the reticulated surface there is a display of iridescence. The egg when first laid is pale green, blending with the natural color of the lower surface of the leaves. With the development of the embryo the color changes to yellowish, which indicates that hatching is imminent. The average dimensions of the eggs measured were 0.378 by 0.648 mm.

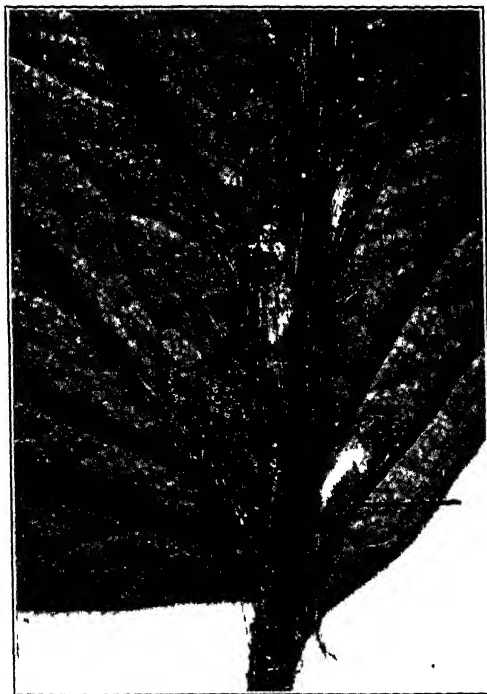


FIGURE 4—Funnel-shaped silky retreat constructed by a young larva of the strawberry leaf roller between two veins on the under surface of a leaf  $\times 2$

#### INCUBATION

The time required for the incubation of the egg depends upon seasonal conditions. In April the eggs hatched in from 14 to 17 days, during June and July in from 6 to 8 days, and during August in from 5 to 9 days. Temperatures for three of these months are shown in Figure 2. Webster (11) found that in Iowa during May, 1919, the eggs hatched in 11.1 days and during July and August, 1915, in from 3 to 12 days.

Dunnam (1) does not mention the month during which his experiments were conducted but states that their "optimum for development" seems to be eight days at 73.02° F.

### THE LARVA

#### RETREATS AND FEEDING AREAS

After emerging from the egg the larvae move slowly over the under surface of the leaf, feeding a little until a suitable place over which to construct their protective silky retreat is found. This tubelike shelter is generally constructed in the angle formed by two veins (fig. 4), at

## THIRD INSTAR

After the second molt the larva measures 3.21 mm in length. In appearance it resembles the larva of the previous stage, and very little change in color or other characters is noticeable. The head width is 0.396 mm. The duration of this instar averages 4 days.

## FOURTH INSTAR

After the third molt the larva measures 4.5 mm in length. The body color ranges from yellowish green to dark green. There are four pairs of light hyaline tubercles or prominences on each body segment, two pairs on the dorsal surface, and a pair on each side. The setae are distributed as in the former stages, but are much longer and more prominent. The width of the head is 0.558 mm. The duration of this instar averages 4.8 days.

## FIFTH INSTAR

After the fourth molt the larva grows rapidly, and when mature measures 12 mm (one-half inch) in length and 1.5 mm in width (Fig 1, B.) The general color is gray-brown above and gray beneath, with the head yellowish brown. In other respects it resembles the previous stage. The duration of this instar averages 6.2 days.

## DURATION OF LARVAL PERIOD

The duration of the larval period of the two summer generations averaged 24.4 days; that of the last generation, which hibernates, averaged 178.3 days.

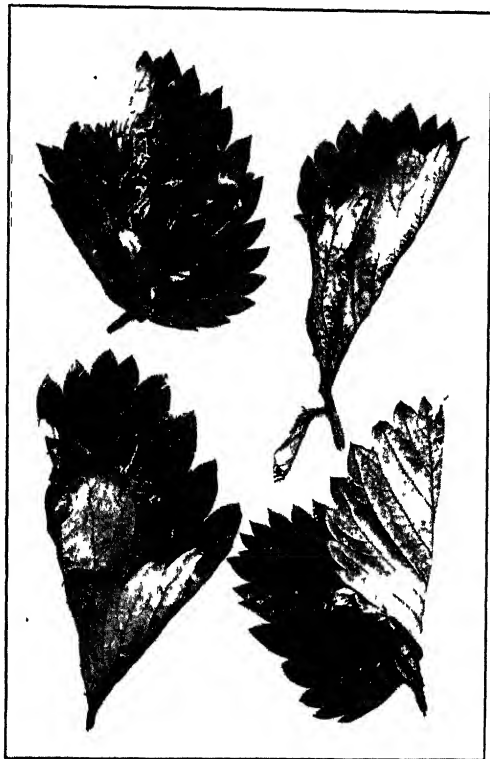


FIGURE 7.—Strawberry leaves folded or rolled in different ways by the larvae of the strawberry leaf roller.  $\times \frac{3}{4}$

## MOLTS

From a large series of experiments it has been determined that in the spring and summer the larvae ordinarily molt four times. During periods of irregular temperature, in the fall, however, larvae that will hibernate continue to feed when the weather is warm and remain inactive when it is cool. As a result of this prolonged feeding the larvae may undergo several additional molts before cold weather makes them entirely inactive. In regard to larvae kept under obser-

simple irregular tubular tent close to the vein. With further growth of the larvae the retreats first made may be abandoned and others constructed in new feeding areas. Very often when two leaves touch or overlap, the larvae web them together at the point of contact and feed within the webbed area.

After the larvae become more than half grown they migrate from the under to the upper surface of the leaves. Here they spin threads of silk attached to either side of the natural depressions formed by the mid veins, and fold the leaves like the wings of a butterfly. (Figs 6 and 7.) Very often one edge of the leaf is folded or rolled. The larvae continue to feed until full grown and eventually transform to pupae within these leaf inclosures. If the larvae are disturbed and dislodged from their inclosures new leaves are rolled or folded.

During its entire growth the larva feeds on either the upper or lower surface of a leaf, leaving the epidermis of the opposite surface intact.

#### DESCRIPTION OF LARVAL STAGES

The descriptions below and the duration given for the different instars are based on observations of 48 individuals of the second generation.

##### FIRST INSTAR

The first-instar larva measures slightly more than 1.5 mm in length, with the head and thorax much wider than the rest of the body. The head measures 0.18 mm in width, is brown, and the tips of the mandibles are red; the rest of the body is pale green. Many long setae are scattered over the head and from four to six are found on the sides of each



FIGURE 6.—The silk strands spun by the larvae of the strawberry leaf roller on the upper surface of the leaf preparatory to rolling or folding the leaf.  $\times 2$

thoracic and abdominal segment, those on the anal segment being the longest. The duration of this instar averages 3.8 days.

##### SECOND INSTAR

After the first molt the larva measures 2.5 mm in length, the head and body being of uniform width. The body color varies somewhat between light green and pale yellow. The setae are arranged as in the first instar. The head across its widest part measures 0.252 mm. The duration of this instar averages 3.4 days.

## DURATION OF PUPAL STAGE

The pupae, as evidenced by their activity, are sensitive to light. They are unable to develop at or above  $40^{\circ}\text{C}$  or at and below  $10^{\circ}\text{C}$ . In April the duration of the pupal period averaged 12.8 days, in June 6.8 days, and in August 6.2 days. (Fig. 2 shows the temperatures during these months.)

Webster (11) states that from meager data the length of this stage in Iowa in April and May was found to be from 14 to 18 days; in the summer months it averaged 6.6 days.

## DURATION OF LIFE CYCLE

Although in breeding experiments under laboratory conditions the average duration of the life cycle of the first generation is 51.4 days and of the second generation 37.9 days (Table 2), it is evident from

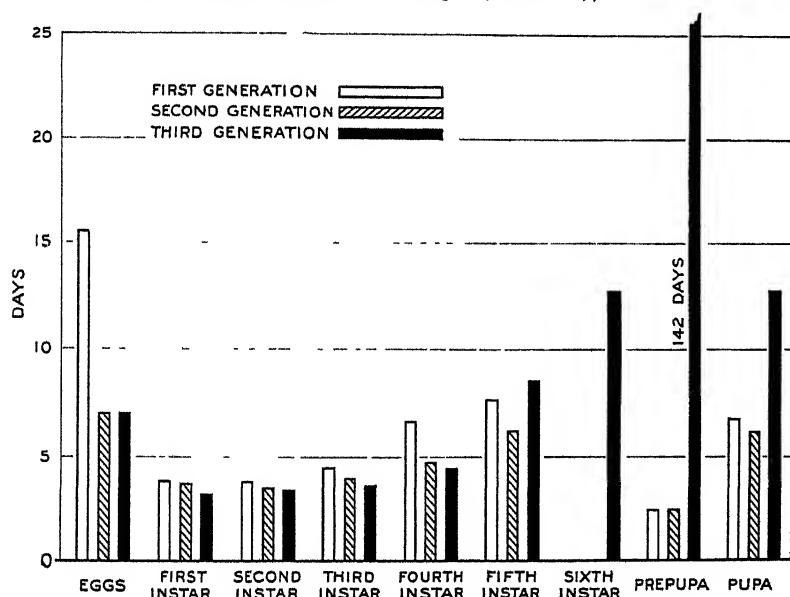


FIGURE 8.—Graph showing the time intervals of the various stages and instars of the three generations of the strawberry leaf roller under laboratory conditions, Riverton, N. J., 1921-22.

field observations that the time may be longer if climatic conditions are unfavorable. In such instances temperature and moisture are probably the controlling factors, since food, in the strawberry beds, is nearly always abundant. The generation that includes the hibernating larvae is of course of longer duration, the average length being 198.1 days. (Fig. 8.)

## NUMBER OF GENERATIONS

Insectary breeding experiments show that, when considered from the first eggs laid, the insect had three complete generations and a partial fourth; and when considered from the last eggs laid it had two complete generations and a partial third. Many larvae of the third generation instead of transforming into pupae during August

vation during the fall as many as seven molts were observed, though five or six were more common. When mature, these larvae were larger and more robust than those of the summer generation, as is shown by the head widths given in Table 1 for the various instars.

TABLE 1 — *Average head-width measurements of larvae of the strawberry leaf roller*

Measurement	First instar	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar
Actual.....	Mm 0 18	Mm 0 252	Mm 0 396	Mm 0 558	Mm 0 882	Mm 1 285
Calculated *.....	18	259	372	535	77	1 108

\* Calculations of head widths were made according to Dvar (2)

Table 2 shows the average number of days between molts of larvae representing three generations. As would be expected when temperature is a contributing factor, the lengths of instars of the first-generation larvae are somewhat greater than those of the second-generation larvae. The longest interval recorded occurred between the last two molts of the larvae about to hibernate.

TABLE 2 — *Average length of stages and instars in the development of the strawberry leaf roller under laboratory conditions, Riverton, N. J., 1921-22*

Generation	Egg stage	First instar	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar	Pre-pupa	Pupa	Total
	Days	Days	Days	Days	Days	Days	Days	Days	Days	Days
First.....	15 5	3 9	3 9	4 5	6 7	7 6	-----	2 5	6 8	51 4
Second.....	7 0	3 8	3 4	4 0	4 8	6 2	-----	2 5	6 2	37 0
Third *.....	7 0	3 6	3 3	3 6	4 5	8 5	12 8	142 0	12 8	198 1

\* Hibernating generation

#### PREPUPAL PERIOD

Upon reaching maturity the larva discharges the waste from the alimentary tract and remains inactive within the folds of the leaf where it has last fed. It may increase the size of its silken retreat after cessation of feeding by spinning a few additional layers of web. The prepupal period in the summer usually lasts from two to three days. The insect hibernates in the prepupal stage, which lasts, on an average, about 142 days. This was determined by observations of the alimentary tract at intervals throughout the dormant period, which showed that no food was taken into the body in the spring prior to pupation.

#### THE PUPA

##### DESCRIPTION

The pupa (fig. 1, C) averages 8.5 mm in length and is yellowish brown when first formed, becoming dark brown several days before the adult emerges. The dorsal surface of each abdominal segment, with the exception of the last three, bears two transverse rows of spines, those of the anterior row of each segment being stouter than those of the posterior row. Each of the last three segments has a single row of spines. In addition, the last segment bears eight slender hairs which are more or less hooked at the tips.

## EFFECT OF TEMPERATURE CHANGES ON HIBERNATING LARVAE

For the purpose of determining the influence which a mild or severe winter might have on a spring outbreak of the strawberry leaf roller, a large number of larvae were collected late in the fall, before freezing temperatures occurred. These larvae were divided into five lots and treated according to the following plan. Lot 1 was kept at a temperature of 24° C, and every day 10 individuals were transferred to a temperature of 10°, kept for a certain period, and then placed at a temperature of 24°; lot 2 was kept at a temperature of 31°, and every day 10 individuals were transferred to a temperature of 10°, kept for a certain period, and then placed at a temperature of 24°; lot 3 was treated like lot 2, except that after exposure to a temperature of 10° the larvae were again placed at a temperature of 31°, lot 4 was subjected to a temperature of 10° for a month and then kept at a temperature of 24°; lot 5 was subjected to a temperature of 10° for a month and then held at a temperature of 31°.

In Table 3 the column of figures listed under each temperature represents the number of days 10 larvae were held at that temperature. Under each lot the first temperature is the initial one. For example, under lot 1 the first line means that the 10 larvae were held for 1 day at 24° C, then for 6 days at 10°, and finally for 5 days at 24°, when some larvae pupated. In the same lot the fourth line means that 10 larvae were held at 24° for 3 days and at 10° for 16 days, when some larvae died.

TABLE 3—*The effect of temperature changes upon hibernating larvae of the strawberry leaf roller*

NUMBER OF DAYS LARVAE WERE EXPOSED AT TEMPERATURES INDICATED (° C)

Lot 1			Lot 2			Lot 3			Lot 4		Lot 5	
24°	10°	24°	31°	10°	24°	31°	10°	31°	10°	24°	10°	31°
1	6	a 5	1	6	a 5	1	11	a 4	30	a 3	30	a 3
2	11	a 9	2	11	a 5	2	13	a 5	30	a 4	30	a 4
3	15	a 15	3	15	a 4	3	10	b 7	30	a 5	30	a 5
3	b 16	---	3	16	a 4	4	14	b 4	30	a 6	30	a 6
4	14	b 8	4	14	b 6	5	12	b 3	30	b 6	30	a 7
5	15	b 5	4	b 14	---	6	13	b 4	30	b 8	30	b 6
5	16	b 3	5	13	b 2	7	b 15	---	30	a 8	30	a 8
8	b 19	---	5	13	b 8	8	16	b 2	30	a 12	30	a 9
9	18	b 5	6	b 13	---	9	19	b 5	---	---	---	---
10	b 18	---	6	16	b 8	10	17	b 4	---	---	---	---
10	b 23	---	7	15	b 3	11	18	b 5	---	---	---	---
13	b 28	---	7	b 16	---	---	---	---	---	---	---	---
14	b 27	---	10	b 23	---	---	---	---	---	---	---	---
16	b 6	---	11	b 25	---	---	---	---	---	---	---	---
17	b 5	---	---	---	---	---	---	---	---	---	---	---
19	b 3	---	---	---	---	---	---	---	---	---	---	---

a Some larvae pupated

b Some larvae died

The results indicate quite clearly that with slight exceptions the mortality was greatest in those larvae activated by a high temperature before they were subjected to a low temperature. This is strikingly revealed in the results obtained from lots 1, 2, and 3, which suffered a mortality of over 90 per cent. In contrast to the above, the larvae of lot 4 were not subjected to a high temperature, and consequently were not rendered active, before exposure to a low temperature. When these larvae were exposed to a high temperature, development continued normally, and they pupated later with a death rate

continued to feed, grow, and molt, and finally passed the winter as full-grown larvae. Other larvae of the same brood transformed into pupae during August, and the moths emerged and deposited eggs. The larvae of this partial fourth generation matured late in the fall and passed the winter successfully. Similar conditions probably occur in the field, and from the finding of larvae of different sizes late in the fall it is supposed that these late-developing larvae came either from the last eggs of the second-brood moths, or from eggs deposited early in the fall by moths of the third generation.

### HIBERNATION

As stated earlier in this discussion, larvae developing in the fall grow much larger than those of the summer generations, and from two to three additional molts occur. It seems evident that this extended period of feeding affords opportunity for the accumulation of a larger amount of adipose tissue, which probably serves the larvae during the period of hibernation. To determine the actual amount of adipose tissue formed, the fat content of the larvae of the summer generations was compared to the fat content of the hibernating larvae. The experimental procedure was as follows: Fifty mature larvae of the summer generations were weighed, macerated, and placed in an oven at 100° C until the material registered a constant weight. The remains were then extracted several times with equal parts of alcohol and ether and the resulting loss in weight recorded. From these data the percentage of fat was calculated. Similar tests were made with the hibernating larvae. The larvae of the summer generations yielded 3.3 per cent fat, the hibernating larvae 8.7 per cent, or more than double the percentage obtained from the summer larvae.

When hibernating larvae were dissected it was noticed that lobed layers of adipose tissue in striking abundance surrounded the alimentary tract. In larvae of the summer generations, however, no such extensive layers of adipose tissue were found.

With the advent of low temperatures in the fall (10° to 15° C), feeding by the larvae ceases, and the waste products in the digestive tract are entirely eliminated. Numerous examinations made by dissecting hibernating larvae at intervals during the winter months showed that the digestive tract remains practically free of food material, with only a slight accumulation of waste material in the rectum. A reduction of about 15 per cent of the normal (83 per cent) water content takes place previous to hibernation, but to prevent further loss of water and to maintain the minimum consistent with successful hibernation it is necessary that the larvae be constantly surrounded by a humid atmosphere. This condition is maintained in nature by the position of the hibernacula in the moist leaf accumulations on the ground. Experiments demonstrated that hibernating larvae placed in cages indoors would not survive the winter unless proper moisture was supplied. Hibernating larvae placed in a large tin container and kept moist passed the winter without injury.

Throughout the winter months hibernating larvae are only slightly dormant and when subjected to a temperature of 27° C. transform to pupae within from three to six days. That the larvae are extremely resistant to cold is shown by their survival in the colder sections of the country when protected by folded leaves only.

N J, more than a dozen species of parasites were reared. A few proved to be new to science, and several species were observed to be of considerable economic importance in the natural control of the strawberry leaf roller. They are listed below in the order of their probable importance. *Macrocentrus ancylivora* Roh, *Cremastus cookii* Davis, *Spilocryptus exannulatus* Cushman, *Exorista pyste* Walk, *Perisierola* sp.,



FIGURE 9.—Parasites of the strawberry leaf roller. A, larva of *Macrocentrus ancylivora* emerging from a leaf-roller larva, B, three larvae of a species of *Perisierola* feeding on a larva of the strawberry leaf roller, C, strawberry leaf-roller larva bearing eggs of *Exorista pyste*, D, cocoon of *Spilocryptus exannulatus* constructed after emergence from a pupa of the strawberry leaf roller. All  $\times 4$ .

*Sympiesis ancylae* Gir, *Dibrachys meteori* Gahan, *D. aeneoviridis* Gir, *Habrocytus* sp., *Pseudacrias (Pleurotropis) sexdentatus* Gir, *Spilochalcis* sp., *Epiurus indagator* Walsh, *Itopectis conquisitor* Say.

*Macrocentrus ancylivora* (fig 9, A) is considered one of the most important of the parasites, since 60 per cent of the larvae of the strawberry leaf roller collected during June and August were found parasitized by this species. It is a large yellow braconid, the body of the

of only 6 per cent. An exposure of less than four days to a temperature of  $31^{\circ}$  C. was apparently not of sufficient duration to activate the larvae, and when they were later subjected to a temperature of  $10^{\circ}$  no injury resulted. On the other hand, when larvae were subjected to a temperature of  $31^{\circ}$  for four days or more, activation and probably development progressed sufficiently to cause injury and death when they were exposed to a temperature of  $10^{\circ}$ , but a sudden shift from  $10^{\circ}$  to  $25^{\circ}$  or  $30^{\circ}$  was not fatal, provided there was no further exposure to temperatures below  $10^{\circ}$ .

From the results here recorded it appears that there is perhaps a particular stage in the development of the larvae at which hibernation may be successful, possibly a stage in which the concentration of certain ingredients permits the protoplasm to resist a low temperature. It is evident, therefore, that a sustained high temperature in the field for a sufficient length of time during the winter followed by a temperature of  $10^{\circ}$  C or lower would be sufficient to cause a high rate of mortality and preclude the possibility of an outbreak of this pest.

When hibernating larvae become activated at a temperature of  $24^{\circ}$  C for a sufficient length of time to cause development to proceed toward pupation, the resulting pupae were found to resist a temperature of  $10^{\circ}$  for only a short interval. This was shown by the following observation. During examinations made when the temperature was  $10^{\circ}$  some pupae which had previously been exposed to a temperature of  $24^{\circ}$  were observed to have proceeded in their development to a point represented by a dark-brown coloration. Under normal conditions moths would have appeared several days later, but further development was checked and death of the pupae followed. Other pupae of the same lot similarly treated made no progress in development and died in the early part of pupal life.

#### EFFECT OF TEMPERATURE ON PUPAL DEVELOPMENT

The rate of development of the pupa was found to be greatly dependent, within certain limits, upon temperature conditions, as shown by the following experiments: Recently formed pupae were divided into six lots and when treated as shown completed development in the number of days specified—

- Lot 1 Pupae held at a constant temperature of  $20^{\circ}$  C completed development in 12 days
- Lot 2 Pupae held at a constant temperature of  $24^{\circ}$  completed development in 12 days
- Lot 3 Pupae held at a constant temperature of  $27^{\circ}$  completed development in 5 5 days
- Lot 4. Pupae held at a constant temperature of  $34^{\circ}$  completed development in 4 days
- Lot 5 Pupae held at a constant temperature of  $40^{\circ}$  died
- Lot 6 Pupae held at a constant temperature of  $10^{\circ}$  died

From these data it is apparent that the limits of development for the pupae probably lie between  $15^{\circ}$  and  $34^{\circ}$  C, with an optimum between  $27^{\circ}$  and  $34^{\circ}$ .

#### NATURAL CONTROL

##### PARASITES

It would appear from the large number of parasites reared from the larval and pupal stages of the strawberry leaf roller that it is ordinarily held in check by natural enemies. In the vicinity of Riverton,

## SUMMARY

The strawberry leaf roller feeds on the foliage of the strawberry, blackberry, and raspberry and may become established in widely separated localities through the transportation and setting out of infested plants.

Fertilized females deposit from 20 to 120 eggs, usually on the under surface of the leaves, and the eggs hatch in from 5 to 17 days.

Until half grown the young larvae feed on the under surface of the leaves protected by silky retreats. They then migrate to the upper surface of the leaves, which they roll or fold, and within these folded leaves they continue feeding and finally pupate. The summer-generation larvae molt four times, and the hibernating larvae may molt six or more times.

The prepupal period in the summer generations lasts from two to three days and in the hibernating generation it lasts throughout the winter. The pupal stage lasts from 6 to 13 days. The life cycle, or developmental period, of the summer generations averaged 51.4 days for the first generation and 37.9 days for the second, and that of the hibernating generation averaged 198.1 days.

Hibernation takes place in the prepupal stage within the rolled or folded strawberry leaves lying on the surface of the ground. An increase in the accumulation of adipose tissue and a reduction of the water content of these larvae precede hibernation.

Experiments indicate that when hibernating larvae are subjected to a temperature of 24° or 31° C. for four days or more and are afterwards placed at a temperature of 10° for a considerable time a high mortality results. If kept first at a temperature of 10° for a considerable length of time and afterwards placed at a temperature of 24° or 31°, pupation occurs and the mortality is very low.

Experiments with pupae indicate that the limits of pupal development are between 15° and 34° C., with an optimum between 27° and 34°.

The strawberry leaf roller is attacked by more than a dozen species of parasites of which the following are the most important: *Macrocentrus ancylivora*, *Cremastus cooki*, *Spilocryptus exannulatus*, and *Exorista pyste*.

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female measuring 4.5 mm in length and having an ovipositor 5.5 mm in length. The male is somewhat smaller, measuring 3.5 mm in length. The biology of this parasite has been described by the writer (4).

*Cremastus cooki* parasitized 15 per cent of the larvae of the strawberry leaf roller collected during June and August. This species is slightly larger than *Macrocentrus ancylirota*, measuring from 6 to 7 mm in length and having an ovipositor about two-thirds the length of the abdomen. Although no studies of its biology were made, observations indicate that its habit of parasitizing the host resembles that of *M. ancylirota*.

*Spillocryptus exannulatus* parasitized from 5 to 10 per cent of the larvae of the strawberry leaf roller collected during June and August. The egg of this parasite is deposited within the larva of the host. The latter, however, is able to develop and pupate, although the parasite larva eventually consumes the contents of the pupa. It then emerges from the pupal remains of the host as a larva and spins about itself a white cocoon (Fig 9, D). The pupal stage lasts from 10 to 14 days. The adult measures 5.5 mm in length, but its ovipositor is only 1.5 mm long. The thorax and posterior part of the abdomen are black, and the anterior portion of the abdomen is yellowish brown.

A species of *Perisierola* (family Bethyilidae) parasitized only from 2 to 5 per cent of the strawberry leaf-roller larvae collected during June. The adult deposits several eggs on the abdomen of the host larva, and the parasitic larvae that hatch from these eggs insert their mouth parts through the epidermis and feed from the exterior upon the internal contents of the host (Fig 9, B). After two or three days the parasitic larvae attain full growth and, without constructing a cocoon about themselves, fasten their anal segments by means of threads of silk to the leaf and transform to pupae in their larval skins, which eventually become jet black. The pupal stage lasts from five to eight days.

The tachinid fly *Exorista pyste* parasitized 5 per cent of the larvae of the host. The adult fly deposits from one to three eggs, either on the thorax or on the last abdominal segment of the host (Fig 9, C). The white, glossy, hemispherical eggs measure 0.456 by 0.27 mm, and their surfaces are reticulated. The lower flattened surface adheres tenaciously to the skin of the host. In hatching, the eggshell splits at one side close to the base, and the parasitic larva bores through the skin into the interior of the host. The host larva, however, is able to mature and transform into the pupal stage, and the parasitic larva, after consuming the contents of the pupa, emerges by breaking through the pupal skin of the host. In many instances the puparium of the parasite was found partly within the empty pupal skin, indicating that pupation had occurred without the parasitic larva entirely emerging from the host. The puparium measures 4 by 1.7 mm and is dark brown. The pupal stage lasts from a week to 10 days. If the host larva bearing eggs of this parasite happens also to be parasitized by *Macrocentrus ancylirota* only the latter develops.

#### PREDACIOUS ENEMIES

The strawberry leaf roller is also attacked by several species of predacious insects. The bugs *Nabis ferus* L. and *Podisus maculiventris* Say and the beetle *Casnomus pennsylvanica* L. were observed feeding on the larvae.

# EFFECTS ON COTTON OF IRREGULAR DISTRIBUTION OF FERTILIZERS<sup>1</sup>

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## INTRODUCTION

In 1929 the joint committee on fertilizer application,<sup>3</sup> the South Carolina Agricultural Experiment Station, and the United States Department of Agriculture joined forces to study the efficiency of fertilizer distributors designed for use in growing cotton. The field tests were conducted at two locations in South Carolina.

A previous paper<sup>4</sup> gives the specifications of the 22 distributors selected for this work, the formulas of the fertilizers used, details of the experiment, and observations obtained in the field and laboratory. The field observations included measurements of the effects of the fertilizers on germination, earliness of blooming, rate of growth, and yield. These results were presented from the standpoint of the efficiency of the distributors.

Earlier studies<sup>5,6</sup> have shown that distributors apply fertilizers unevenly along the row, owing to cycles of delivery and other causes. They differ widely, however, in the kind and extent of these variations in delivery, as may be seen in Figure 1. It would be reasonable to suppose that such differences in distribution would produce corresponding effects on the crop, but insufficient evidence is available to show the character or magnitude of these effects.

The present paper gives a statistical analysis of some of the results previously published, in order to show the differences in the effects of fertilizers on cotton when applied uniformly and with typical degrees of irregularity.

## PLANTING

The results of the applications made by hand and by distributors Nos. 1, 2, 4, 5, and 8 in the previous study<sup>7</sup> were selected for this work, because the conditions surrounding them were substantially the same for each test, except that the fertilizers were applied with different degrees of variability of distribution. The fertilizers were applied in open furrows, over which raised seed beds were formed and dragged to a uniform height. Later examination of the placements showed that the fertilizers were in bands 2 or 3 inches wide and 3 inches below the surface. The seeds were planted 1 inch below the surface at the rate of 1 every inch. Thus in each instance the fertilizer was placed in narrow bands 2 inches below the seed.

<sup>1</sup> Received for publication Nov. 13, 1931, issued May, 1932.

<sup>2</sup> Credit is due Avis J. Peterson, Fertilizer and Fixed Nitrogen Investigations, Bureau of Chemistry and Soils, for a large number of the calculations required in this work.

<sup>3</sup> Composed of representatives of the American Society of Agronomy, the American Society of Agricultural Engineers, the National Association of Farm Equipment Manufacturers, and the National Fertilizer Association.

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TABLE 1.—The number of cotton plants per 36-foot row above ground on the dates shown for different degrees of variability of distribution of 4-8-4 and 12-24-12 fertilizers applied at the rates indicated

Kind of fertilizer	Method of application	Quantity of fertilizer applied and number of plants standing in—											
		Norfolk coarse sand, planting No 6, planted May 9				Cecil sandy clay loam, planting No 7, planted May 14				Cecil sandy clay loam, planting No 8, planted May 24			
		Plants standing on—		Fertilizer applied	Pounds per acre	Plants standing on—		Fertilizer applied	Pounds per acre	Plants standing on—		Fertilizer applied	Pounds per acre
		May 22	May 29			May 21	May 31			May 31	June 7		
4-8-4	Hand.....	800 41±15	343±6	Number	800	306±8	289±8	Number	800	272±4	365±3	Number	332±4
	Distributor No 1.....	794 64±7	368±6	8	636	311±9	315±3	287±2	683	272±4	365±3	332±4	419±6
	Distributor No 4.....	762 35±7	366±4	9	962	306±5	310±1	306±5	707	275±3	394±8	401±7	401±7
	Distributor No 5.....	521 108±14	334±5	10	536	309±7	295±3	315±1	708	278±3	413±9	491±10	491±10
	Distributor No 8.....	735 98±24	355±10	15	730	321±7	321±2	330±4	707	271±7	370±9	372±9	402±13
None	Distributor No 2.....	767 123±24	354±7	56	800	319±6	342±4	318±8	692	272±9	396±12	416±9	416±9
	Hand.....	287 71±21	366±4	335±5	287	311±6	306±3	324±11	267	270±8	420±7	367±15	367±15
	Distributor No 4.....	305 69±14	279±23	267±27	235	306±8	296±13	327±10	304	277±1	374±4	370±18	370±18
	Distributor No 1.....	20 307	273±21	241±28	303	306±8	304±6	345±8	287	289±15	377±16	378±10	378±10
	Distributor No 5.....	320 148±10	302±11	268±12	303	298±7	317±5	332±8	355	249±18	383±4	385±4	385±4
12-24-12	Distributor No 8.....	31 155±23	288±8	260±8	293	326±7	307±9	327±11	286	218±8	356±4	349±11	349±11
	Distributor No 2.....	260 181±21	322±12	285±12	240	322±6	308±3	319±5	280	274±12	398±14	397±11	397±11
	Hand.....	257±14	361±4	340±1	257±14	314±3	340±9	341±5	257±14	280±10	389±9	389±9	389±9
	Distributor No 4.....	18 305	279±23	267±27	235	311±6	306±3	324±11	267	270±8	420±7	367±15	367±15
	Distributor No 1.....	20 307	273±21	241±28	303	306±8	296±13	327±10	304	277±1	374±4	370±18	370±18

\* Variability of distribution based on 1-foot intervals of delivery.

A coefficient of variability ( $V$ ) may be used as a measure of the degree of irregularity of distribution of the fertilizer. It is calculated from the weights of material in consecutive parts of the row.  $V$  was calculated for each machine from the weights of fertilizer delivered in 40 consecutive 1-foot sections of row, and the values obtained cover the usual range in practice. Special precautions were used in making the hand applications, and, although strictly speaking they could not be perfect, they may be considered uniform ( $V=0$ ). These determined values of  $V$  will be used hereafter to designate the different degrees of variability of distribution.

Eight plantings, each consisting of a set of the several degrees of variability of distribution of the fertilizers, were made at intervals during a period of seven weeks. The first six were made at the Sand-

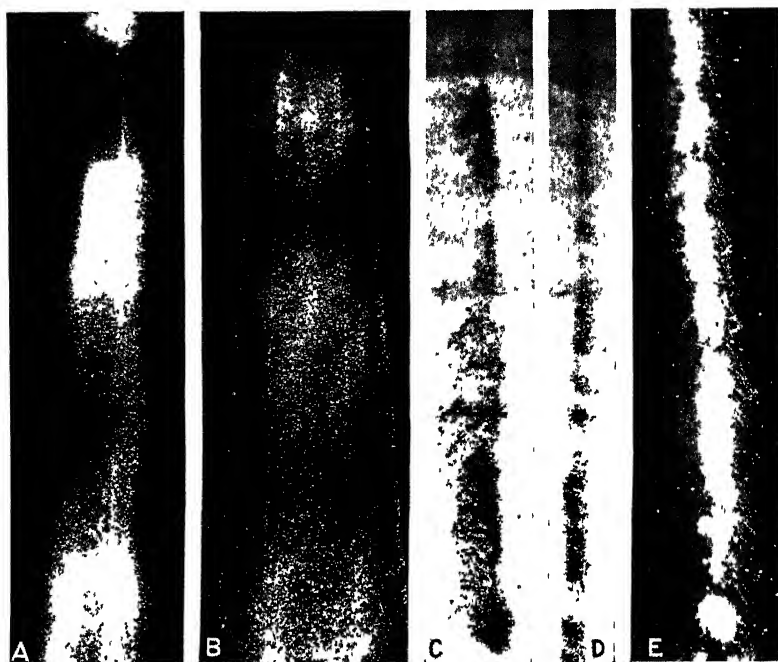


FIGURE 1—Characteristic distribution of fertilizers by various commercial machines. The coefficients of variability on a 1-foot basis are as follows: A, 66; B, 36; C, 30; D, 12; and E, 8.

hills experiment station near Columbia in Norfolk coarse sand. Five of these were destroyed by adverse weather before complete results could be obtained, and therefore no detailed report will be made of them. The last two were made in Cecil sandy clay loam at Clemson College. Each test consisted of four replicate plots in different parts of the field. The plots were single 36-foot rows staked out of the middle of longer rows.

The rates of application of the 4-8-4 and 12-24-12 fertilizers were as close to 800 and 267 pounds per acre, respectively, as the various machines were capable of giving. The rates actually obtained were determined in each test and are shown in Table 1. The rates were such that the same amounts of nitrogen, phosphoric acid, and potash were applied in each fertilizer.

Standard deviations ( $\sigma$ ) of the number of plants per foot of row were calculated, therefore, to determine the effect of each degree of variability on uniformity of stand. Each value of  $\sigma$ , presented in Table 2, is based on 144 feet of row.

TABLE 2—Standard deviations ( $\sigma$ ) of the numbers of cotton plants per foot of row for different degrees of variability of distribution (V) of fertilizers

Kind of fertilizer	V	Standard deviations of numbers of plants per foot of row standing in—							
		Norfolk coarse sand, planting No 6		Cecil sandy clay loam, planting No 7		Cecil sandy clay loam, planting No 8		Averages of the differences from the checks for the three tests	
		$\sigma$	Differences from checks	$\sigma$	Differences from checks	$\sigma$	Differences from checks		
-8-4-----	0	2 09±0 08	+0 05	2 80±0 15	+0 03	3 88±0 15	+0 37	+0 15	
	8	1 85± 07	- 19	3 15± 12	+ 38	3 62± 04	+ 11	+ 10	
	9	2 20± 09	+ 25	3 02± 17	+ 25	3 59± 16	+ 08	+ 19	
	10	2 10± 08	+ 06	2 91± 31	+ 14	3 76± 17	+ 25	+ 15	
	15	2 30± 09	+ 26	3 03± 26	+ 26	3 98± 16	+ 47	+ 33	
None-----	56	2 03± 10	+ 59	3 44± 27	+ 67	4 10± 03	+ 59	+ 62	
		2 04± 08		2 77± 18		3 51± 10			
12-24-12-----	0	2 38± 09	+ 24	1 82± 16	- 09	3 12± 11	- 21	- 02	
	18	2 48± 10	+ 34	2 09± 15	+ 18	3 58± 38	+ 25	+ 26	
	20	2 60± 10	+ 46	2 28± 27	+ 37	3 86± 15	+ 53	+ 45	
	24	2 83± 11	+ 69	2 34± 05	+ 43	3 70± 13	+ 37	+ 50	
	31	2 78± 11	+ 64	2 56± 29	+ 65	3 72± 21	+ 39	+ 56	
	66	2 83± 11	+ 69	2 74± 29	+ 83	4 08± 11	+ 75	+ 76	
None-----		2 14± 09		1 91± 20		3 33± 19			



FIGURE 2—Irrregular stand of cotton due to irregular distribution of 4-8-4 fertilizer applied at a rate of 800 pounds per acre in a 2-inch band 2 inches below the seed. The spacing of the bunches of plants in the foreground corresponds closely to the length of the delivery cycle (5.4 feet). The correspondence, however, in other plots was not so close as that shown here.

A certain amount of irregularity of spacing is due to irregular planting of the seed, which varied somewhat from an average of about 12 seed per foot. This variation was greatest in plantings Nos 7 and 8. Part of the irregularity of stand was also due to differences in soil and viability of the seed. We may assume that all such effects on stand will be measured approximately by the value of  $\sigma$  for the unfertilized checks. These causes should have had about the same effects

The same machines applied both fertilizers, but from the values of  $V$  it will be seen that the degree of variability was greater in each case when the smaller quantity of the concentrated fertilizer was applied by the same machine.

With the greatest degree of irregularity, the fertilizer was deposited in the manner shown at A in Figure 1, and the high points in the delivery cycle were about 17 feet apart where roughly ten times as much fertilizer was deposited as at the low points. With the smallest degree they were 3.8 feet apart, and the delivery rate varied from 20.6 to 28.6 g per foot.

## GERMINATION

### NUMBER OF SEED GERMINATING

The sixth planting was made on May 9, and at this time the coarse sand contained 5.3 per cent moisture. From May 17 until the middle of June rains fell every few days. Consequently the soil moisture was favorable during most of the germination period. The fertilizers slightly delayed germination on this soil, as shown by the results of counts in Table 1. Pearson's correlation coefficient,  $r$ , between the numbers of seedlings aboveground at the first count and the corresponding coefficients of variability is  $0.734 \pm 0.089$ . There was a definite tendency, therefore, for more seed to germinate promptly where the fertilizer was irregularly applied. At the last count there were no significant differences in the number of seedlings due to irregular distribution. The percentage of germination was lower on all the plots fertilized with the 12-24-12 mixture in this planting.

Plantings Nos. 7 and 8 were made on Cecil sandy clay loam containing 15.47 per cent moisture on May 14 and 11.6 per cent on May 24. A rain amounting to 0.81 inch fell on May 27, but during the remainder of the germination period the rainfall was very slight. There was no significant delay in germination on this soil, and the percentage of germination was the same with both fertilizers.

The results of other experiments on these soils where the same amounts of fertilizer were placed 2 inches below the seed, although not given here, also indicate that in light sandy soils the percentage germination is likely to be somewhat lower when fertilizers are uniformly distributed than when irregularly distributed, but on heavy clay soils no significant differences occur as a rule.

### SPACING OF THE SEEDLINGS

Plants appeared simultaneously all along the rows on the uniformly fertilized plots, but they came up in bunches at the low points in delivery on the Norfolk sand plots that were fertilized in a cyclic manner. Figure 2 shows irregular germination due to the same cause in a later experiment on Ruston sandy loam. Although the total number of seedlings finally appearing above ground in any one group of tests was approximately the same for each degree of variability of distribution, the seedlings were more uniformly distributed along the row on the uniformly fertilized plots. These plots had at least one seedling in each foot of row, and in a few cases as many as 3 consecutive feet were bare on plots where  $V=56$  and 66. The last count for each planting was made after new seedlings ceased to appear, and the number of plants in each foot of row was counted separately.

fertilized ones, and they also varied less in size, as will be seen by comparing Figures 3 and 4

The results of bloom counts are given in Table 4. Although some of the figures are erratic, uniform distribution of fertilizer appears to be of real value in promoting early blooming.

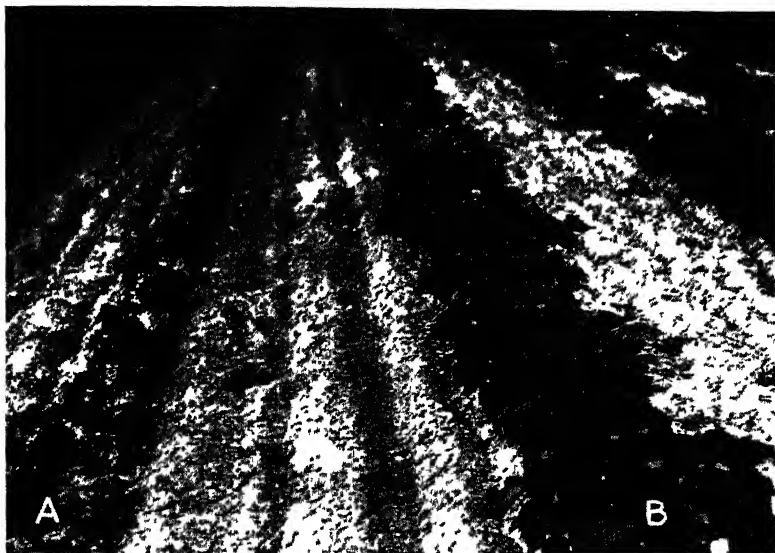


FIGURE 3—A, Unfertilized cotton plants used as checks, B, uniformly fertilized plants

TABLE 4—Number of cotton-plant blooms per plot as related to various degrees of variability of distribution (*V*) of fertilizers

Fertilizer and rate of application per acre	<i>V</i>	Number of blooms per plot on plants standing in—											
		Norfolk coarse sand, planting No 6				Cecil sandy clay loam, planting No 7				Cecil sandy clay loam, planting No 8			
		July 18-24	July 25-31	Aug 1-3	Total	July 26-Aug 1	Aug 2-8	Aug 9-10	Total	July 26-Aug 1	Aug 2-8	Aug 9-10	Total
		—	—	—	—	—	—	—	—	—	—	—	—
800 pounds 4-8-4 <sup>a</sup>	0	14	60	27	101	53	122	60	235	43	115	64	222
	8	5	54	28	87	50	81	41	172	38	95	57	190
	9	4	40	24	74	47	105	35	187	40	103	53	196
	10	8	61	26	95	45	93	50	188	27	83	45	155
	15	8	60	26	94	48	102	46	196	23	105	54	182
None	56	8	56	33	97	52	88	46	186	28	100	52	180
	0	0	0	1	1	13	59	34	106	4	25	13	42
	6	6	40	19	65	50	84	63	197	23	105	28	156
	18	4	41	21	66	43	92	45	180	20	92	34	146
	20	5	31	15	51	38	96	50	184	22	83	27	132
267 pounds 12-21-12 <sup>a</sup>	24	3	45	19	67	41	96	53	190	16	70	27	113
	31	4	50	18	72	24	87	32	143	4	29	14	47
	66	3	26	12	41	32	99	50	181	13	66	26	105
	0	0	0	1	1	15	48	31	94	2	22	7	31
	None	0	0	1	1	32	48	31	94	2	22	7	31

<sup>a</sup> Approximately. For exact rates see Table 1

#### YIELDS

The crops were harvested in two pickings, and the weights of seed cotton converted to an acre basis are presented in Table 5.

on each  $\sigma$  for the same group, but if the fertilizer at certain points prevented seeds from germinating, the corresponding value of  $\sigma$  will be higher. Consequently the differences between the values of  $\sigma$  for the fertilized and corresponding check plots are an indication of the effect of the irregular distribution of the fertilizer on stand. Irregular distribution of the fertilizer increased the value of  $\sigma$  by a maximum of about 0.8. This of course means that a number of seed were prevented from germinating at several points in each plot where excessive amounts of fertilizer were deposited. When the differences between  $\sigma$  for the fertilized and unfertilized plots are averaged for the three plantings, as shown in the last column of Table 2, and correlated with the corresponding values of  $V$ ,  $r$  equals  $0.920 \pm 0.030$ .

Thus, although irregular distribution had no marked effect on the percentage of germination in these tests, it did have a measurable effect on the uniformity of spacing of the plants, and the effect was proportional to the degree of variability of distribution. Fertilizers when uniformly applied had no very significant effect on either percentage of germination or uniformity of stand.

### EARLY GROWTH AND BLOOM

After the plants were well started a tapeline was laid beside the rows, and the plants were thinned by hand wherever possible to a stand of one plant every 6 inches. The height of a dozen plants from each plot was measured at this time. The heights given in Table 3 were obtained by averaging the mean figures from the four replicates.

TABLE 3.—Average heights of cotton plants as an indication of rate of growth for different degrees of variability of distribution ( $V$ ) of fertilizers

Fertilizer and rate of application per acre	$V$	Average heights of plants (inches) standing in—					
		Norfolk coarse sand, planting No 6		Cecl sandy clay loam, planting No 7		Cecl sandy clay loam, planting No 8	
		June 11	July 9	June 15	July 3	June 15	July 3
800 pounds, 4-8-4 <sup>a</sup> .....	0	7 1	13 1	12 2	17 8	7 4	15 1
	8	7 1	12 3	10 7	16 8	7 3	12 7
	9	7 3	10 8	10 7	15 0	7 2	12 3
	10	6 8	12 6	10 8	14 6	7 7	11 5
	15	7 0	12 6	9 9	14 4	7 4	11 6
	56	6 8	11 2	9 3	15 4	7 4	11 6
None.....		4 5	7 5	7 1	11 5	6 0	10 1
	0	7 2	14 6	11 0	15 9	7 1	11 1
	18	6 6	12 6	8 9	13 6	7 3	11 5
	20	6 6	9 6	9 1	13 7	6 9	12 5
	24	5 8	10 5	9 5	15 2	7 3	12 5
	31	6 1	10 9	9 2	14 8	7 4	11 8
267 pounds, 12-24-12 <sup>a</sup> .....	66	6 5	10 2	9 2	14 1	6 9	12 5
		4 5	7 4	7 4	11 1	6 3	10 2
	0	7 2	14 6	11 0	15 9	7 1	11 1
	18	6 6	12 6	8 9	13 6	7 3	11 5
	20	6 6	9 6	9 1	13 7	6 9	12 5
	24	5 8	10 5	9 5	15 2	7 3	12 5
None.....	31	6 1	10 9	9 2	14 8	7 4	11 8
	66	6 5	10 2	9 2	14 1	6 9	12 5
		4 5	7 4	7 4	11 1	6 3	10 2
	0	7 2	14 6	11 0	15 9	7 1	11 1
	18	6 6	12 6	8 9	13 6	7 3	11 5
	20	6 6	9 6	9 1	13 7	6 9	12 5
Mean probable errors of heights.....		$\pm 18$	$\pm 37$	$\pm 17$	$\pm 32$	$\pm 10$	$\pm 32$
$r$ between $V$ and heights of plants.....		$- 501$	$- 612$	$- 685$	$- 413$	$- 285$	$- 152$
Probable errors of $r$ .....		$\pm 146$	$\pm 122$	$\pm 103$	$\pm 161$	$\pm 179$	$\pm 188$

<sup>a</sup> Approximately, for exact rates see Table 1

A rather definite correlation is shown between  $V$  and the average rate of growth by the values of  $r$  at the bottom of Table 3. In general the uniformly fertilized plants grew more rapidly than the unevenly

As stated previously, every effort was made to distribute the ordinary and concentrated fertilizers at rates of 800 and 267 pounds per acre, respectively, and the rates actually obtained are given in Table 1. These rates, as well as those used in determining the coefficients of variability of distribution, were sufficiently different from the standards in some cases to influence the yields. It was therefore desirable, if possible, to correct both sets of figures for these discrepancies.

The South Carolina experiment station<sup>8</sup> had been studying the effect of varying the rate of application of 4-8-4 fertilizers on the yields of cotton for a number of years, under conditions very similar to those of the present experiments. These data were used in an attempt to correct the present yields in the following manner. Two curves, which may be called the master curves, were drawn to represent the relationship shown by the experimental evidence between rate of application and yields. The yield to be corrected and its corresponding check yield were then plotted on the same chart with its master curve, and a smooth curve of the same shape as the master curve was drawn through



FIGURE 4—Irrregularly fertilized cotton plants. Groups of tall plants occur at intervals approximately corresponding in length to those of the cycles of delivery of the 4-8-4 fertilizer ( $V=55$ )

the two points. The corrected yields (shown in Table 5) were determined by the points at which these curves crossed the 267 and 800 pounds per acre lines in the graph.

The hand distribution was assumed to have a coefficient of variability of zero, as was explained previously. In all other cases  $V$  could be corrected because it had been determined for each machine at two or more rates of application. The coefficients obtained in each case were plotted against rate of application and the several points were connected by a smooth curve. Corrected coefficients of variability were read from these curves and are also given in Table 5. It will be noticed that the check yields are the same for both fertilizers in planting No. 6 but are different in plantings Nos. 7 and 8. This is probably because Nos. 7 and 8 were located on terraced land, which is characteristic of this section of the Cotton Belt. In the seventh planting the 12-24-12 group of tests was on the lower part of a terrace,

<sup>8</sup> BUIE, T. S., and WARNER, J. D. COTTON FERTILIZER EXPERIMENTS. S. C. Agr. Expt. Sta. Bul. 245, 32 p., illus. 1928.

TABLE 5.—Yields of seed cotton (pounds per acre) when fertilizers were applied with various degrees of variability of distribution (V)

Kind of fertilizer	V, corrected to uniform rate of application	Quantity of seed cotton (pounds per acre) yielded by plants standing in—						Means of total yields of three plantings		
		Norfolk coarse sand, planting No. 6		Cecil sandy clay loam, planting No. 7		Cecil sandy clay loam, planting No. 8		Total corrected to uniform rate of fertilization	Crude data	Corrected to uniform rate of fertilization
		First picking Oct 10 <sup>a</sup>	Total	Total corrected to uniform rate of fertilization	First picking Oct 14 <sup>b</sup>	Total	Total corrected to uniform rate of fertilization			
4-5-1	0	832±63	861±65	861	940±1	983±1	983	1,255±21	1,036	1,036
	7.8	781±26	821±23	825	773±23	884±15	921	1,070±11	925	935
	9.3	670±58	736±50	758	914±24	1,003±29	960	1,093±12	944	946
	9.6	735±23	794±24	786	841±38	986±55	970	1,102±32	961	967
None	15.1	623±23	662±21	695	846±35	940±33	932	1,137±37	913	930
	55.3	660±52	698±51	710	763±18	872±13	872	967±14	846	861
	30±5	30±5	65±7	—	463±19	594±24	—	495±27	355	—
	0	640±64	681±62	681	1,074±19	1,265±40	1,265	1,093±6	1,013	1,013
12-24-12	18.0	488±42	533±43	501	840±18	1,061±27	1,076	960±32	857	839
	19.7	540±53	587±58	545	1,077±38	1,256±46	1,210	943±27	929	893
	24.0	437±74	487±74	420	907±67	1,107±81	1,052	882±35	809	774
	30.6	415±40	415±40	446	726±39	889±59	961	840±67	715	758
None	66.2	356±41	404±40	414	929±42	1,071±47	1,080	674±46	716	733
	32±7	32±7	64±12	—	508±33	1,681±36	—	297±41	347	—

<sup>a</sup> Second picking, Nov 8<sup>b</sup> Second picking, Nov 6

ings and the original values of  $V$  were used first. When the data for the 4-8-4 fertilizer, the 12-24-12, and both simultaneously, are correlated,  $r = -0.840 \pm 0.081$ ,  $-0.665 \pm 0.153$ , and  $-0.704 \pm 0.098$ . For the corrected figures  $r = -0.894 \pm 0.055$ ,  $-0.810 \pm 0.095$ , and  $-0.795 \pm 0.072$ , respectively. The latter coefficients are better, and their size indicates that if these experiments were repeated under the same conditions, differences in yield might be predicted from the degrees of variability of distribution of the fertilizer by means of a regression equation with an accuracy of about 70 per cent.

#### TIME OF MATURITY

Both fertilizers greatly hastened maturity, as shown by comparisons between the results from fertilized and unfertilized plots. The uniformly applied fertilizers not only produced more cotton, but in nearly every instance they also hastened maturity to a greater extent than the same fertilizers applied irregularly. In the few cases of irregular distribution, where a greater percentage of the crop was mature at the first picking, the total produced was small in proportion to that produced by the corresponding uniformly applied fertilizer.

#### DISCUSSION

In this study an ordinary fertilizer applied in a band 2 inches below the seed at planting time at the recommended rate by machines giving a representative range of irregular distribution increased the average yield of three plantings on two different soils by 548 pounds of seed cotton per acre over the yields of the unfertilized checks. When the same quantity of the same fertilizer was evenly distributed the average yield was 652 pounds greater than the checks, or 19 per cent greater than the average for the machines. The corresponding figures when the concentrated fertilizer was used are 452 and 666. In this case 214 pounds of seed cotton to the acre or 47 per cent more crop was produced. The 4-8-4 and 12-24-12 fertilizers were 37 per cent and 73 per cent more efficient in increasing yields, respectively, when uniformly applied than when applied with the greatest degree of irregularity. It is realized that under other conditions the differences might not be so striking, and further studies will be reported elsewhere.<sup>11</sup> Nevertheless, it appears that the degree of variability of distribution of fertilizers is a factor of importance in the production of cotton.

Any additional expense that might be incurred by the farmer, in obtaining proper equipment, or by manufacturers in refinement of fertilizer distributors and in improvement of the drillability of fertilizers, to secure more uniform distribution would undoubtedly be small compared to the benefits that might be expected.

#### SUMMARY

Ordinary and concentrated fertilizers at rates of 800 and 267 pounds per acre, respectively, were applied uniformly by hand and with five typical degrees of irregular distribution by commercial distributors in barrow bands 2 inches below cottonseed in Norfolk coarse sand and

<sup>11</sup> The later report has been published since this was written. See CUMMINGS, G. A., MEHRING, A. L., LEWIS, G. H., and SACHS, W. H. PROGRESS REPORT ON MECHANICAL APPLICATION OF FERTILIZERS TO COTTON IN SOUTH CAROLINA, 1930. U. S. Dept. Agr. Circ. 192, 31 p., illus. 1931.

and the 4-8-4 group was on the higher part of the slope. In the eighth planting these conditions were reversed. The growing conditions on the lower part of such terraces are probably somewhat different from those on the upper.

The uniformly applied fertilizer is clearly superior to that where  $V=7.8$  or 18, and these in turn are decidedly superior to the most irregularly applied fertilizer in increasing yields. The differences in the six cases where the foregoing comparisons can be made are in the same direction and greater than the probable error in every case and in several instances from five to seven times as large.

To get a better idea of how much reliance should be placed on these differences in yield, odds were calculated by Student's method,<sup>9</sup> using the tables of odds given by Love.<sup>10</sup> The yields of those replicate plots that may be paired for this purpose are given in Table 6.

TABLE 6—Yields of seed cotton in pounds per acre of plots used to determine odds by Student's method

Kind of fertilizer	Corrected coefficient of variability $V$	Yield (pounds per acre) of replicate plots on—							
		Norfolk coarse sand, planting No 6				Cecil sandy clay loam, planting No 7		Cecil sandy clay loam, planting No 8	
4-8-4-----	0	1,108	791	946	599	996	991	1,301	1,213
	7.8	813	779	762	934	817	923	1,064	1,077
	55.3	956	584	600	651	868	854	1,013	1,006
12-24-12-----	0	970	548	501	705	1,349	1,181	1,107	1,082
	19.7	888	253	742	467	1,376	1,247	890	962
	66.2	481	220	532	384	1,226	1,022	893	691

The figures in any line may be paired with those in the same vertical columns of either of the other two lines given for the same fertilizer. Accordingly, eight pairs of figures from three plantings on two widely different types of soil were used in calculating the significance of the differences of the average yields. The differences in yields between the pairs may be considered as due primarily to the method of application and secondarily to soil differences, and those between the yields in the same line primarily to soil variance. The odds, as calculated by Student's method, that the uniform applications are really superior to the most irregular applications in increasing yields, are 488 to 1 and 529 to 1 for the 4-8-4 and 12-24-12 fertilizers, respectively. The corresponding odds that uniform distribution is better than that with a coefficient of variability of 7.8 in increasing yields are 9 to 1, and those that the distribution with a coefficient of 19.7 is superior to that with a coefficient of 66.2 are 22.8 to 1. In every case the odds are in favor of the greater degree of uniformity of distribution. This fact, together with the size of the odds, makes it practically certain that the major part of the average differences in yield are due to the degree of variability of distribution of the fertilizer.

Coefficients of correlation were computed for each fertilizer to determine the extent to which fluctuations in yield correspond to those of  $V$ . The averages of the actual yields obtained in the three plant-

<sup>9</sup> ANONYMOUS. THE PROBABLE ERROR OF A MEAN. By Student. *Biometrika* 6 1-25, illus. 1908.

<sup>10</sup> LOVE, H. H. A MODIFICATION OF STUDENT'S TABLE FOR USE IN INTERPRETING EXPERIMENTAL RESULTS. *Jour. Amer. Soc. Agron.* 16 68-73. 1924.

# ACETIC ACID AND PYROLIGNEOUS ACID IN COMPARISON WITH FORMALDEHYDE AS SOIL DISINFECTANTS<sup>1</sup>

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## INTRODUCTION

Formaldehyde is one of the most commonly used of soil disinfectants. Its efficacy against fungi in the soil is well established, but it has at least two faults. The cost of the treatment is relatively high and the time which must be allowed to elapse between treatment and seeding, if the treatment is not to injure germination, is sometimes objectionable. The principal object of the work here described was to find a soil disinfectant as effective against fungi, lower in cost, and less injurious to seeds than is formaldehyde.

In earlier experiments, the results of which have been described by the writer,<sup>2</sup> 10 to 12 per cent acetic acid used as a soil disinfectant was found to protect seedlings from damping off. Similar results with acetic acid as a soil disinfectant have since been secured by other investigators.<sup>3 4</sup>

The evidence presented in this paper verifies the conclusions previously reached in regard to acetic acid and serves as a basis for comparing acetic acid, pyroligneous acid, and formaldehyde as soil disinfectants. In the present paper various dilutions and rates of application of acetic acid, pyroligneous acid, and formaldehyde are compared with each other as regards prevention of damping off (caused by species of *Pythium* and *Rhizoctonia*), effect on seed germination, and effect on growth (dry weight) of plants.

## METHODS

The soil used is a water-deposited fine sandy loam. In all cases (except in a forest nursery, to which reference is made below) manure was composted with this soil as in ordinary greenhouse practice. The soil prior to the application of the treatments contained water to the extent of 60 per cent of its water-holding capacity (except as otherwise indicated).

For each series of experiments, all seeds were sown the same day, in order that the effects of the treatments on growth of plants might be compared. Treatments were in triplicate and 900 seeds of beet, cucumber, and lettuce were used for each. Seeds were sown at the rate of 50 per linear foot. After germination was completed and damping off had ceased, seedlings were thinned so as to leave the same number per linear foot, in order that competition between plants should not interfere with the effects of soil treatment on growth.

<sup>1</sup> Received for publication Oct. 26, 1931, issued May, 1932. Contribution No. 126 of the Massachusetts Agricultural Experiment Station.

<sup>2</sup> DORAN, W. L. ACETIC ACID AS A SOIL DISINFECTANT. Jour Agr. Research 36: 269-280, illus. 1928.

<sup>3</sup> ANDERSON, P. J., SWANBACK, T. R., and STREET, O. E., and others. DAMPING OFF OF YOUNG TOBACCO SEEDLINGS. Conn Agr. Expt. Sta. Bul. 311: 269-270, illus. 1930.

<sup>4</sup> SLATE, W. L. BOTANY. Conn Agr. Expt. Sta. Bul. 318: 757. 1930.

Cecil sandy clay loam A statistical analysis was made of measurements of the crops produced

The results indicate that a larger number of seed germinate promptly but the seedlings are more irregularly spaced along the row when fertilizers are irregularly applied than when they are uniformly distributed

More rapid and uniform growth, earlier blooms, earlier maturity, and larger yields of cotton were produced by uniform applications than by irregular ones The extent of these effects was decidedly significant and varied with the degree of irregularity of distribution.

soil to which formaldehyde 1:50 (1 gallon of formaldehyde with 49 gallons of water) had been applied 10 days before seeding.

All damping off of the seedlings of beet, cucumber, and lettuce was also prevented by acetic acid 1.19 per cent applied to soil 5 or 13 days before seeding and at the rate of  $2\frac{1}{2}$  quarts per square foot. In other experiments conducted by the writer damping off has been prevented equally well by acetic acid 1.19 per cent applied to soil at the rate of 2 quarts per square foot. Thus used, the cost of the acetic-acid treatment, per unit area of soil, is about three-fourths of the cost of soil treatment with formaldehyde.

In connection with this use of acetic acid, experiments with vinegar as a soil disinfectant were undertaken, for vinegar is, of course, readily available to every farmer and gardener. The user of vinegar is relatively certain of obtaining a standardized product as regards content of acetic acid, for, according to the standard adopted in enforcing the Federal food and drug act,<sup>6</sup> cider or apple vinegar (also grape or wine vinegar, malt vinegar, sugar vinegar, glucose vinegar, and spirit vinegar) must contain not less than 4 g of acetic acid per 100 cc. Such vinegar when diluted by the addition of  $2\frac{1}{2}$  parts of water to 1 part of vinegar (by volume) will, therefore, contain in this dilution about 1.16 per cent acetic acid. Vinegar thus diluted was applied to soil (at the rate of 2 quarts per square foot), and 10 days later tobacco seeds were sown in the treated soil and in soil not treated. Seeds germinated well (equally well in both cases), and as may be seen by reference to Figure 1, there was no damping off in soil to which vinegar had been applied, although the disease was severe in the untreated soil.

In earlier experiments,<sup>7</sup> seedlings of white spruce were protected against damping off by 1.12 per cent acetic acid applied to soil at the rate of 1.64 quarts per square foot seven days before seeds were sown. With the object of improving upon this method for use in forest nurseries, acetic acid (0.47 to 0.80 per cent) was applied to seed beds, at the rate of three-fourths of a quart per square foot, immediately after the seeds of red or Norway pine (*Pinus resinosa* Sol.) were sown. Damping off was severe in the untreated soil. Living seedlings (in 4 square feet of each seed bed) were counted three months after the date of seeding. The results are recorded in Table 2. Seed germination was improved, damping off was controlled, and the number of seedlings living three months after seeding was increased most (more than 700 per cent) by 0.8 per cent acetic acid (4.2 pounds of 80 per cent acetic acid diluted with water to 50 gallons) applied to the soil at the rate of three-fourths quart per square foot immediately after seeding. The merits of this treatment for the prevention of damping off in a forest nursery are the successful control of damping off, the harmlessness to seeds (of red pine), the relatively small amount of water, and therefore of labor, needed, and the avoidance of delay between soil treatment and seeding.

<sup>6</sup> UNITED STATES DEPARTMENT AGRICULTURE, FOOD AND DRUG ADMINISTRATION. DEFINITIONS AND STANDARDS FOR FOOD PRODUCTS. U. S. Dept. Agr., Food and Drug Admin. Ser. and Regulat. Announc., Food and Drug No. 2 (second revision), 19 p. 1931.

<sup>7</sup> DORAN, W. L. Op. cit. (See footnote 2).

Three weeks later the plants were pulled, washed, and dried to constant weight.

The dilutions of pyroligneous acid, acetic acid, and formaldehyde which were applied to soil are recorded in Table 1, as are also the intervals of time which elapsed after soil treatment and before seeding. Except as is otherwise indicated, the diluted chemicals were applied to soil at the rate of 2½ quarts per square foot.

Undistilled pyroligneous acid was used. As described by the manufacturers, it was made by the destructive distillation of hardwood (beech, birch, and maple) in sealed retorts. Pyroligneous acid was found to have certain advantages over either acetic acid or formaldehyde as a soil disinfectant, and the results are accordingly presented. Further work will, however, be necessary before the observed effects of pyroligneous acid on fungi in the soil can be traced to each of its several constituents, since, according to Hawley,<sup>5</sup> pyroligneous acid is not a chemical compound but contains a number of constituents, including acetic acid, methyl alcohol, formaldehyde, and furfural.

TABLE 1—Effects of formaldehyde, acetic acid, and pyroligneous acid on seed germination, damping off of seedlings, and dry weights of plants

Soil treatment	Time interval between soil treatment and seeding	Germination of seeds of different plants			Plant seedlings which damped off			Dry weights of 100 plants	
		Beet <sup>a</sup>	Cucumber	Lettuce	Beet	Cucumber	Lettuce	Beet	Cucumber
	Days	Number	Per cent	Per cent	Per cent	Per cent	Per cent	Grams	Grams
Check.....	10	83	60	15	10	61	45	16.61	30.50
Formaldehyde 1 50.....	10	129	84	45	0	0	0	20.78	91.85
Acetic acid 1 19 per cent.....	5	129	100	72	0	0	0	111.27	-----
Do.....	13	138	85	60	0	0	0	20.79	-----
Acetic acid, 1 78 per cent.....	13	40	-----	-----	0	-----	-----	20.58	-----
Pyroligneous acid, 1 100.....	12	-----	-----	-----	1	29	-----	35.00	-----
Pyroligneous acid, 2 100.....	1	120	92	56	0	0	0	-----	-----
Do.....	2	136	89	63	0	0	0	-----	113.50
Do.....	3	107	80	80	0	16	0	-----	-----
Do.....	5	86	93	75	17	4	7	-----	-----
Do.....	13	140	-----	-----	6	-----	-----	23.69	-----
Pyroligneous acid, 3 100.....	1	128	72	49	0	0	0	-----	-----
Do.....	2	83	93	57	2	0	0	-----	118.93
Do.....	3	108	81	73	0	0	0	-----	-----
Do.....	6	92	80	53	0	0	0	-----	-----
Do.....	13	139	-----	-----	3	-----	-----	22.66	-----
Pyroligneous acid, 4 100.....	1	128	97	60	0	0	0	-----	-----
Do.....	2	108	73	56	0	0	0	-----	125.28
Do.....	3	155	97	68	0	0	0	-----	-----
Do.....	5	124	72	-----	0	0	0	-----	-----
Do.....	7	124	83	45	0	0	0	-----	-----
Do.....	13	141	-----	-----	0	-----	-----	17.26	-----
Pyroligneous acid, 5 100.....	13	-----	-----	-----	0	0	-----	-----	38.73
Pyroligneous acid, 10 100.....	13	-----	-----	-----	0	0	-----	27.40	-----

<sup>a</sup> Number of seedlings which came up for each 100 beet seed balls sown.

<sup>5</sup> HAWLEY, I. F. WOOD DISTILLATION. 141 p., illus. New York, 1923.

## EFFECTS OF SOIL DISINFECTANTS ON DAMPING OFF OF SEEDLINGS

The average percentages of seeds which germinated, seedlings which damped off, and dry weight of plants are recorded in Table 1. Damping off was severe in untreated soil, for in it 40 per cent of the beet seedlings, 61 per cent of the cucumber seedlings, and 45 per cent of the lettuce seedlings damped off. There was no damping off in

TABLE 2—*Effects of soil treatment with various strengths of acetic acid on the damping off of the seedlings of red pine*

Percentage of acetic acid applied to soil	Living seedlings per square foot (3 months after seeding)	Increase in seedlings per unit area as compared with check	Percentage of acetic acid applied to soil	Living seedlings per square foot (3 months after seeding)	Increase in seedlings per unit area as compared with check
0 (check)-----	Number 37	Per cent	0 33-----	Number 236	Per cent 538
0 47-----	163	340	0 80-----	301	713
0 67-----	265	616			

All damping off of seedlings listed in Table 1 was prevented by pyroligneous acid 10:100, 5:100, and 4:100.<sup>8</sup> Pyroligneous acid 3:100 prevented all damping off of cucumber and lettuce seedlings, but there was a little damping off of beet seedlings, 2 to 3 per cent, in soil to which pyroligneous acid 3:100 had been applied. In these experiments, pyroligneous acid 4:100 was as effective in preventing all damping off as was formaldehyde 1:50 or acetic acid 1.19 per cent. In other experiments, damping off of seedlings was controlled equally well by pyroligneous acid  $3\frac{1}{2}$ :100, applied to soil at the rate of 2 quarts per square foot. Thus used, the cost of soil treatment with pyroligneous acid  $3\frac{1}{2}$ :100 was about 58 per cent of the cost, per unit area, of soil treatment with formaldehyde 1:50.

In the experiments recorded in Table 1 pyroligneous acid 1:100 or 2:100 did not prevent all damping off of the seedlings of beet, cucumber, and lettuce. These concentrations are considered too dilute to be dependable, although in some cases pyroligneous acid 2:100 has given adequate protection. When pyroligneous acid 2:100 was applied to soil at the rate of 2 quarts per square foot seven days before tobacco seeds were sown there was, as may be seen by reference to Figure 1, no damping off of tobacco seedlings grown in soil so treated, although the disease was severe in untreated soil.

#### EFFECTS OF SOIL DISINFECTANTS ON SEED GERMINATION

In the untreated soil 60 per cent of the cucumber seeds and 45 per cent of the lettuce seeds germinated and 83 beet seedlings came up for each 100 beet seed balls sown. Much of this poor germination was due to the decay of seeds resulting from the attack of damping-off fungi in the soil.

The germination of the seeds of beet and cucumber was improved, and the germination of the seeds of lettuce was unaffected by formaldehyde 1:50 applied to the soil 10 days before seeding.

The germination of the seeds of beets was injured by 1.78 per cent acetic acid, and on the basis of these and other experiments it is not considered necessary to use a greater concentration of acetic acid than 1.2 per cent for soil disinfection.

The germination of these seeds was improved as much, or more, by 1.19 per cent acetic acid as by formaldehyde, and this was the case in the experiments represented in Table 1, whether acetic acid

<sup>8</sup> Dilutions of pyroligneous acid to which reference is made in the text and in Table 1 are indicated as number of parts (by volume) of pyroligneous acid in 100 parts of water.

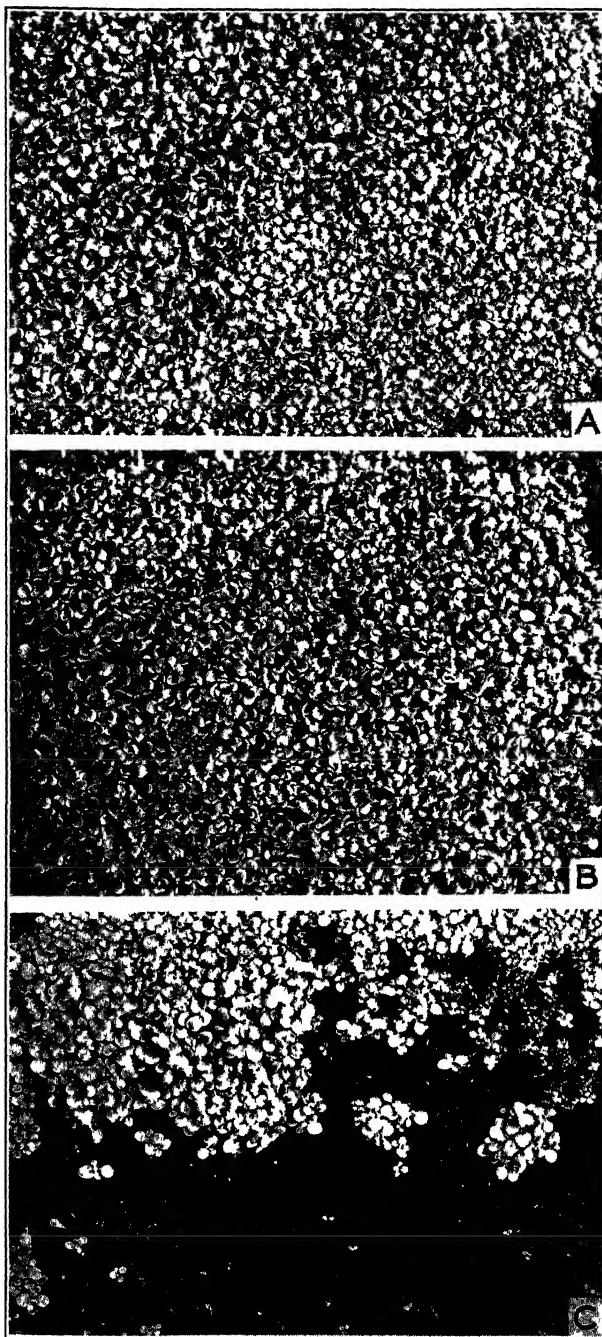


FIGURE 1.—Tobacco seedlings protected against damping off by soil treatment with pyroligneous acid and with vinegar. A, Pyroligneous acid, 2 100, applied to soil at the rate of 2 quarts per square foot (7 days before seeding), B, vinegar 1 part with water 2½ parts (by volume) applied to soil at the rate of 2 quarts per square foot (10 days before seeding), C, check, no disinfectant used

The dry weight of plants was increased by the application of formaldehyde to the soil 10 days before seeding. Acetic acid 1.19 per cent was no less beneficial, for in soil to which it had been applied the dry weight of cucumber seedlings was increased more and the dry weight of beet seedlings was increased as much as by formaldehyde. In these, as in other experiments, the application of acetic acid 1.19 per cent, at the rate of 2 or 2½ quarts per square foot of soil, was followed by an improved growth of plants, and the improvement in growth was ordinarily as great as that associated with the use of formaldehyde. Even the use of acetic acid 1.78 per cent, a concentration which may injure seed germination, was without any injurious effect on the growth of beets in soil to which this treatment had been applied 13 days before seeding.

There was no retarding of growth of plants in soil to which pyroligneous acid 1:100, 2:100, 3:100, 4:100, 5:100, and 10:100 had been applied, even though the treatments with pyroligneous acid 2:100, 3:100, and 4:100 were applied to soil only one or two days before seeding. Dry weight of cucumber seedlings was increased more by pyroligneous acid 3:100 or 4:100 applied to soil two days before seeding than by formaldehyde 1.50 applied to soil 10 days before seeding. The dry weight of beet seedlings was also increased by these treatments, and the increase, as compared with that which followed the use of formaldehyde, was greater with pyroligneous acid 3:100 and less with pyroligneous acid 4:100. In these and in other experiments by the writer the beneficial effect of soil treatment with pyroligneous acid on growth of plants was no less than with formaldehyde.

#### SUMMARY

Acetic acid was as safe and as effective a soil disinfectant as formaldehyde, and the cost of soil disinfection with acetic acid was less than with formaldehyde. Damping off of seedlings (of beet, cucumber, and lettuce) was prevented without injury to seed germination and with benefit to growth of plants by soil treatment with 1.19 per cent acetic acid (1 gallon of 56 per cent acetic acid or 2¾ quarts of 80 per cent acetic acid with water to total 50 gallons), applied at the rate of 2 to 2½ quarts per square foot of soil. An application of 2 quarts per square foot was usually enough.

Best results with acetic acid against soil-borne fungi in tobacco seed beds have been secured when the soil was treated in the fall rather than in the spring.

With acetic acid, as with formaldehyde, it was necessary that there be some interval of time, usually 10 days, between soil treatment and seeding; otherwise seed germination was injured.

Damping off of seedlings (of tobacco) was prevented with no injury to germination by vinegar 1 part diluted with water 2½ parts (by volume), applied to soil at the rate of 2 quarts per square foot 10 days before seeding.

Seedlings of red or Norway pine were protected against damping off, and germination was not injured by acetic acid 0.8 per cent (equal to 6 pounds of 56 per cent acetic acid or 4.2 pounds of 80 per cent acetic acid with water to total 50 gallons), applied to soil at the rate of three-fourths of a quart per square foot at the time of seeding.

was applied to the soil 5 days or 13 days before seeding. In other experiments by the writer seed germination has sometimes been injured, however, if seeds were planted in less than 10 days after the application of acetic acid to soil, and this is considered the minimum time interval before seeding which is usually safe after soil treatment with either acetic acid or formaldehyde.

In the case of tobacco seed beds the delay which must follow the application of these soil treatments in the spring is sometimes objectionable. This is of course avoidable by applying such treatments in the fall; and, as observed by the writer, best results have been secured in tobacco seed beds when the soil was disinfected with acetic acid in the fall rather than in the spring.

Chemical soil disinfection of tobacco seed beds in the spring may have another disadvantage, for if the soil is very wet, as it often is at that season, neither acetic acid nor formaldehyde as ordinarily applied always prevents all damping off. Earlier investigators<sup>9</sup> have suggested applying formaldehyde to tobacco seed beds in the fall rather than in the spring if the soil is likely to be very wet. Their conclusions are supported by the results of experiments by the writer, in which formaldehyde 1:50 (2 quarts per square foot) was less effective in preventing damping off when applied to water-saturated soil than it was when applied to soil which, previous to treatment, contained water to the extent of 50 per cent of its water-holding capacity.

As may be seen by reference to Table 1, the germination of the seeds of beet, cucumber, and lettuce was in most cases improved and in no case injured by soil disinfection with pyroligneous acid 2:100, 3:100, and 4:100. Seeds were uninjured even though the interval between soil treatment with pyroligneous acid 3:100 or 4:100 and seeding was reduced to one or two days. In other experiments it was, however, found unsafe to shorten this interval to less than one day or to apply pyroligneous acid 2:100, 3:100, or 4:100 to living plants. When these treatments were applied, at the rate of 1 quart per square foot, to seedlings of beet, cucumber, and lettuce which had begun to damp off, the plants were severely injured. When pyroligneous acid 3:100 was applied to soil at the rate of 2 quarts per square foot, at the same time that the seeds of pepper, lettuce, cucumber, and tomato were sown, the germination of the seeds of cucumber and tomato was unaffected, but the germination of the seeds of pepper and lettuce was injured.

These observations lead to the conclusion that pyroligneous acid like acetic acid or formaldehyde should be applied to the soil before sowing most seeds. But in the experiments above described and with the seeds used it was safe to apply pyroligneous acid to the soil as late as one day before seeding, and this is a matter of convenience which is sometimes important in practice.

#### EFFECTS OF SOIL DISINFECTANTS ON DRY WEIGHT OF PLANTS

By reference to Table 1 the dry weight of beet and cucumber seedlings in each of the several soil treatments may be compared with the dry weight of plants in untreated soil. There was considerable increase in dry weight of plants following most treatments, and it was greater with cucumber than with beet.

<sup>9</sup> SELBY, A. D., HOUSER, T., and HUMBERT, J. G. HOW TO DISINFECT TOBACCO PLANT BEDS FROM ROOT-ROT FUNGUS (TRICHLAVIA). Ohio Agr. Expt. Sta. Circ. 156, 5-8, illus. 1915.

# THE INFLUENCE OF PHOSPHATES ON THE PHOSPHORIC ACID CONTENT OF THE PLANT<sup>1</sup>

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## REVIEW OF PREVIOUS INVESTIGATIONS

During the last 15 or 20 years much attention has been given to the matter of improving pastures in this country as well as in Europe. This in turn has led to renewed interest in the mineral requirements of animals and to a study of the so-called mineral deficiency in pastures and feeding materials. Orr<sup>2</sup> cites McDougall's estimate that the value of the grassland products annually consumed in Great Britain is roughly 426,000,000 pounds. He further says:

Considerably more than half of these is imported, the imports forming nearly a quarter in money value of our total imports \* \* \*. In the British Isles there are 34,000,000 acres of grass of one kind or another, and there are numerous tracts of pasture lands in the Domimons and Colonies. As pasture is the raw material of many of the primary necessities of life, for which there is a constant market, the improvement and better exploitation of these pastures is one of the surest methods of securing the stable prosperity of the Empire.

Recently much interest has centered in the influence of fertilizers on the mineral composition of the plant. An attempt has been made to find out to what extent the low value of certain pastures is due to mineral deficiencies in the soil. In this connection Russell<sup>3</sup> says

Phosphate starvation markedly affects the composition of crops, lowering their nutritive value to animals and their special quality values to men. Over large areas of the world, soils are very deficient in phosphate. Those occurring in parts of South Africa carry a natural herbage which causes deficiency diseases in cattle; the affected animals devour bones with great eagerness, even putrefying bones when the deficiency is pronounced, so that they become liable to a particular ptomaine poisoning. The obvious remedy is to feed the cattle with bone meal. Similar diseases occur in Australia, where also the arable land shows astonishing benefits from small dressings of superphosphate. In the Romney Marsh the best fattening pastures are richer in phosphates than the poorer ones, this is generally true of England and France.

Investigations in New Zealand have shown that basic slag and superphosphate applied to land that is deficient in phosphoric acid resulted in increasing the percentage of phosphoric acid in grasses. The influence of the superphosphate was most marked in the early spring, but its effect was noted throughout the season. Orr and Scherbatoff<sup>4</sup> have reported at length on some of the problems relating to the influence of mineral fertilizers on the composition of pasture grasses. Their investigations led them to conclude that

the mineral composition of pasture is affected by the composition of the soil on which it grows, and that the alteration of the composition of the soil by the application of fertilizers increases the mineral content of the pasture, the increase

<sup>1</sup> Received for publication Oct. 26, 1931, issued May, 1932.

<sup>2</sup> ORR, J. B., and SCHERBATOFF, H. MINERALS IN PASTURES AND THEIR RELATION TO ANIMAL NUTRITION 1-2. London, 1929.

<sup>3</sup> RUSSELL, E. J. SOIL CONDITIONS AND PLANT GROWTH. Ed. 5, p. 81. London, New York [etc.] 1927.

<sup>4</sup> ORR, J. B., and SCHERBATOFF, H. Op. cit., p. 48.

Pyroligneous acid 3:100 to 4:100 applied to soil at the rate of two quarts per square foot protected seedlings from damping off, and this treatment did not injure the germination of the seeds of beet, cucumber, and lettuce even when it was applied to the soil as late as one day before seeding. Soil treatment with pyroligneous acid resulted in an increase in the dry weight of plants. Per unit area of soil treated, the cost with pyroligneous acid was less than with either formaldehyde or acetic acid. Pyroligneous acid was as effective a soil disinfectant as formaldehyde or acetic acid, and safer and cheaper than either.

course of the 5-year rotation. The crop was analyzed for phosphoric acid with the results shown in Table 1. In this case plants grown in the cylinders without treatment showed the lowest percentage of phosphoric acid. Those with minerals or minerals and nitrate of soda showed a distinct increase in phosphoric acid, while those that received minerals, nitrate of soda, and manure showed an increase of as much as 40 per cent. A smaller but distinct increase was noted in the oats grain where minerals and nitrate were used on field plots (Table 8). The difference between those with lime and those with lime and green manure is scarcely significant.

TABLE 1—*Phosphoric acid content of oats (grain and straw) and corn (forage) grown in cylinders with different fertilizer treatments, 1930*

Cylinder No	Fertilizer treatment	Phosphoric acid content (per cent) of crops grown on—			
		Limed plots (B's)		Limed plots with green manure added (C's)	
		Oats	Corn	Oats	Corn
1	No fertilizer.....	0.978	0.860	0.937	0.868
2	Minerals *.....	1.237	.929	1.174	1.192
3	Minerals and manure.....	1.330	1.365	1.330	1.296
7	Minerals and nitrate of soda.....	1.162	1.122	1.145	1.157
8	Minerals and double nitrate of soda.....	1.150	1.019	1.128	1.019
9	Minerals, nitrate, and manure.....	1.375	1.145	1.318	1.110
10	Minerals, double nitrate, and manure.....	1.336	1.122	1.342	1.019
17	Minerals and ammonium sulphate.....	1.133	1.088	1.168	1.088
	Average.....	1.213	1.081	1.193	1.094

\* Minerals = 640 pounds of superphosphate and 320 pounds of muriate of potash per acre

CYLINDER EXPERIMENT ON THE INFLUENCE OF NITROGEN, PHOSPHORIC ACID AND POTASH ON THE PHOSPHORIC ACID CONTENT OF BARLEY

In growing barley in Sassafras silt loam in cylinders, phosphoric acid in the form of superphosphate was used in three different quantities, with a single and also a double portion of potash, and with different nitrogenous materials furnishing equivalent amounts of nitrogen. The fertilizer treatment on this soil was begun in 1923. The 1930 crop was harvested just before maturity, and determinations were made on the grain and straw together. The results are shown in Table 2. The double and triple phosphate treatments produced slight increases in the phosphoric acid content of barley as compared with the single treatment. The differences between the single and double potash treatments are not significant. In every case the plants from the cylinders that did not receive nitrogen showed a higher percentage of phosphoric acid in the dry matter than did those that received nitrogen. The plants in the cylinders that received nitrate of soda showed a higher percentage of phosphoric acid than did those receiving the other nitrogenous materials. With slight exceptions, the percentages varied from about 0.7 to a little over 1 per cent  $P_2O_5$ . This soil originally (1922) contained about 0.11 per cent  $P_2O_5$ .

being most marked in poor soils. The increase is due partly to the fact that the individual plants are enriched, and partly to the fact that the fertilizers promote the growth and spread of species of plants which are naturally richer in minerals.

Studies made in various parts of the United States have shown that soils which are naturally rich produce grasses high in mineral constituents. It has also been shown that when phosphates are applied to poor soils the phosphoric acid content of the crop is generally increased. In studying this problem, several investigators have noted that the mineral treatment may distinctly influence the type of vegetation. It has likewise been noted that the composition of the vegetation depends more or less upon the date of cutting. Very young grass may contain a high percentage of phosphorus, but on account of the small yield in the early part of the season the total phosphorus that could be obtained by grazing cattle may be small.

Crowther and Ruston<sup>5</sup> found that in the majority of cases the percentage of phosphoric acid decreased up to and including the third cutting. Their results have been confirmed by others, although work done at the New Jersey Agricultural Experiment Station indicates a somewhat higher percentage of phosphoric acid in the mixed herbage of July, August, and September than was found in herbage cut in the early part of the season.

#### EXPERIMENTAL WORK

As a further contribution to this subject it seemed worth while to make a study of the phosphate content of crop samples from the cylinder experiments and the soil-fertility plots of this station. The original cylinder soils have received definite fertilizer treatment for 32 years, and the field plots for 22 years.

If mineral fertilizers influence the mineral composition of the plant, surely crops grown on soils without phosphate or with definite phosphate treatment for so long a time should give some evidence of such treatment. Therefore phosphoric acid has been determined in a number of crop samples from the field and cylinder experiments in which phosphates have been used for a number of years. Determinations have been made on the following crops. Wheat and oats, both grain and straw, corn, grain and stover, corn forage; timothy hay, rye, grain and straw, young rye (samples collected in the late fall), soybean hay, bailey, grain and straw, barley (grain and straw together); rape, potatoes. Tables showing the different phosphate treatments and the percentage of phosphoric acid in the crop are given. Unless otherwise specified, nitrogen was used in quantities equivalent to 320 pounds of nitrate of soda an acre and potash in quantities equivalent to 320 pounds an acre.

#### CYLINDER EXPERIMENT WITH OATS AND CORN

Oats and corn were grown in Penn loam soil in cylinders without fertilizer and with different fertilizing materials, this soil having been under cultivation and treatment for about 30 years. The soils of one series were limed at intervals of 5 years, while those of the other series were limed and also produced two legume green-manure crops in the

<sup>5</sup> CROWTHER, C., and RUSTON, A. G. THE INFLUENCE OF TIME OF CUTTING UPON YIELD AND COMPOSITION OF HAY. *Jour. Agr. Sci. [England]* 4: 305-317. 1912.

TABLE 3—*Phosphoric acid content of corn forage grown on soils varying in mechanical composition, with and without fertilizer (cylinder experiment), 1930*

Fertilizer treatment <sup>a</sup> (pounds per acre)	Dry matter in and phosphoric acid content of corn forage grown on—									Average		
	Loam soil			Loam soil 80 per cent, and sand 20 per cent			Loam soil 60 per cent, and sand 40 per cent					
	Dry mat- ter		Phosphoric acid	Dry mat- ter		Phosphoric acid	Dry mat- ter		Phosphoric acid	Dry mat- ter		Phosphoric acid
	<i>Gm</i>	<i>Per cent</i>	<i>Gm</i> <sup>b</sup>	<i>Gm</i>	<i>Per cent</i>	<i>Gm</i> <sup>b</sup>	<i>Gm</i>	<i>Per cent</i>	<i>Gm</i> <sup>b</sup>	<i>Gm</i>	<i>Per cent</i>	<i>Gm</i> <sup>b</sup>
Without fertilizer.....	150	0.681	1.021	135	0.762	1.031	108	0.682	0.735	131	0.708	0.929
200.....	201	546	1.095	200	664	1.324	176	593	1.049	192	601	1.156
500.....	254	446	1.130	277	593	1.637	255	431	0.945	262	490	1.237
1,000.....	373	468	1.744	405	517	2.099	376	491	1.863	385	492	1.902
Average.....	245	535	1.248	254	634	1.523	229	549	1.148	243	573	1.306

<sup>a</sup> The fertilizer used analyzed 16 per cent,  $\text{NH}_3$ , 18 per cent  $\text{P}_2\text{O}_5$ , and 16 per cent  $\text{K}_2\text{O}$  made from urea, high-grade superphosphate, and muriate of potash. Half the fertilizer was applied at planting, and half near the end of July.

<sup>b</sup> Grams removed from soil.

#### CYLINDER EXPERIMENT ON THE INFLUENCE OF PHOSPHATES ON THE PHOSPHORIC ACID CONTENT OF RYE, SOYBEAN HAY, AND BARLEY

In cylinder experiments rye was grown on two types of soil, Sassafras loam and Portsmouth loam, with varying amounts of superphosphate and also with raw rock phosphate in equivalent amounts. Phosphoric acid determinations were made on grain and straw, with the results shown in Table 4. In nearly all cases the grain and straw from the cylinders that received the heavier applications of superphosphate showed some increase in phosphoric acid over those that received no phosphate treatment.

With the raw rock phosphate the increases over the no-phosphate treatment were slight. There was little difference between the phosphoric acid content of samples from the limed and unlimed sections.

Soybeans for hay were also grown on these two soils and on a third soil, Colts Neck loam, with the same phosphate treatment. Results are shown in Table 5 for the 1923 crop and in Table 6 for the 1930 crop. Determinations of phosphoric acid in rape grown on Colts Neck loam, with the same fertilizer treatment as the soybeans, are also shown in Table 6.

When 100 and 200 pounds of superphosphate were applied to the acre there was very little increase in the phosphoric acid content of the dry matter, but when 500 and 1,000 pounds of superphosphate were applied to the acre there was nearly always some increase in the phosphoric acid content of the dry matter.

TABLE 2—*Phosphoric acid content of barley when fertilized with different nitrogenous materials, and with different quantities of phosphoric acid and potash, 1930*

Nitrogen treatment	Phosphoric acid content (per cent) of barley grown with—					
	Single $P_2O_5$ treatment and—		Double $P_2O_5$ treatment and—		Triple $P_2O_5$ treatment and—	
	Single $K_2O$ treatment	Double $K_2O$ treatment	Single $K_2O$ treatment	Double $K_2O$ treatment	Single $K_2O$ treatment	Double $K_2O$ treatment
No nitrogen.....	1 038	0 894	1 090	1 113	1 136	1 060
Nitrate of soda.....	923	796	980	958	814	808
Sulphate of ammonia.....	704	710	825	681	814	813
Dried blood.....	745	652	849	831	883	935
$\frac{1}{3}$ nitrogen from each of above three.....	750	687	774	808	882	837
Average.....	781	711	857	820	848	848

## CYLINDER EXPERIMENT ON THE INFLUENCE OF PHOSPHATE ON THE PHOSPHORIC ACID CONTENT OF CORN FORAGE GROWN ON SOILS VARYING IN MECHANICAL COMPOSITION

Corn forage was grown on soils varying in mechanical composition and with varying amounts of fertilizer, and samples of the dry material were analyzed for phosphoric acid. The results of the work are given in Table 3. The average phosphoric acid content of the dry matter for the crop grown on loam soil was 0.535 per cent, for the crop on loam soil with 20 per cent of sand the average was 0.634 per cent, and for the crop on loam soil with 40 per cent of sand it was 0.549 per cent. The average phosphoric acid content of dry matter of the crop grown in cylinders without fertilizer was slightly over 0.7 per cent. With fertilizer at the rate of 200 pounds an acre the average was 0.6 per cent, and with fertilizer at the rate of 500 and 1,000 pounds an acre it was about 0.5 per cent  $P_2O_5$ . It will be noted that the dry matter of the forage grown in cylinders with 20 per cent of sand showed a higher percentage of phosphoric acid than did that of forage grown on loam soil or on loam soil with 40 per cent of sand. In the fertilizer-treatment experiments, the forage grown in cylinders receiving no fertilizer had a higher average percentage of phosphoric acid than did that grown in cylinders receiving fertilizer. Forage in cylinders receiving fertilizer at the rate of 200 pounds an acre had a higher average percentage of phosphoric acid than did that in cylinders receiving 500 and 1,000 pounds of fertilizer an acre. This is not in agreement with other work reported here. Attention has already been called to the fact that in a number of cases heavy applications of superphosphate resulted in some increase in the phosphoric acid content of the crop. In this case, however, the yield of dry matter was very much increased as the amount of fertilizer applied was increased, and this resulted in a proportionate increase in the amount of phosphoric acid removed in the crop. It is therefore possible that this increased demand for phosphoric acid drew so heavily on the available supply in the soil as to cause a reduction in the percentage in the dry matter of the crop. This seems the only explanation for a lowered percentage in the dry matter where the phosphate application was increased.

TABLE 6—*Phosphoric acid content (per cent) of soybean hay and rape (dry) grown on different types of soil under different phosphate treatments, 1930*

Phosphate treatment (pounds per acre)	Phosphoric acid content (per cent) of crops							
	Soybean hay grown on—						Rape (dry matter) grown on—	
	Colts Neck loam		Sassafras sandy loam		Portsmouth loam		Colts Neck loam	
	A*	B	A	B	A	B	A	B
No phosphate.....	0 605	0 652	0 439	0 468	0 595	0 566	0 894	0 885
100, superphosphate.....	640	739	427	560	617	658	900	986
200, superphosphate.....	664	672	468	546	617	710	966	780
500, superphosphate.....	706	718	557	632	721	675	958	853
1,000, superphosphate.....	762	640	530	569	715	744	910	1 003
Average.....	693	692	496	577	668	697	934	906

\* A and B = duplicate treatments

With raw rock phosphate such increases as are noted were slight and in a number of cases there was no increase. Lime seems to have had little influence one way or the other. With the exception of the soybean hay on Sassafras sandy loam in 1930, the type of soil seems to have had little influence. In this connection it may be explained that the Colts Neck loam is exceptionally high in phosphoric acid—about 0.8 to 1 per cent, the Sassafras sandy loam is rather low in phosphoric acid, whereas the Portsmouth loam also contains a high percentage, though not so high as the Colts Neck loam.

In 1923, barley was grown on Colts Neck loam with and without lime, with the phosphate treatments outlined above. Phosphoric acid was determined in the grain and straw, with the results shown in Table 7. In this case neither the phosphate nor the lime had a pronounced influence on the percentage of phosphoric acid in the crop. In a number of cases the crop without phosphate showed as high or a higher percentage of phosphoric acid as the crop that received 1,000 pounds of superphosphate an acre.

#### PHOSPHORIC ACID CONTENT OF GRAIN AND HAY IN FIELD EXPERIMENTS

In the nitrogen-availability field experiments which were started in 1908, certain plots receive no fertilizer, others receive superphosphate only, and others superphosphate and muriate of potash, and still others a complete fertilizer. Phosphoric acid has been determined in timothy hay and also in oats and wheat grain from certain of these plots, with the results shown in Table 8. It is at once apparent that the phosphorus treatment had little influence on the phosphoric acid in the timothy hay, and if averages be considered, about the same must be said of the oats and wheat grain. With the complete fertilizer (minerals and nitrate of soda), the oats grain shows a somewhat higher percentage of phosphoric acid than where no fertilizer is used, but the differences are not great.

TABLE 4 — *Phosphoric acid content (per cent) of rye grown on different types of soil under different phosphate treatments, 1923*

## SUPERPHOSPHATE SECTION

Phosphate treatment (pounds per acre)	Phosphoric acid content (per cent) of rye grown—							
	With lime on—				Without lime—			
	Sassafras loam		Portsmouth loam		Sassafras loam		Portsmouth loam	
	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw
No phosphate.....	0 803	0 159	0 822	0 180	0 799	0 104	0 770	0 115
100, superphosphate.....	777	138	765	179	871	122	905	154
200, superphosphate.....	886	143	900	174	952	159	890	151
500, superphosphate.....	902	185	936	213	952	154	936	167
1,000 superphosphate.....	1 001	190	900	195	1 045	174	921	180
Average .....	892	164	875	190	955	152	913	163

## RAW ROCK PHOSPHATE SECTION

No phosphate.....	0 884	0 135	0 853	0 195	0 890	0 169	0 843	0 148
Rock phosphate equivalent to 100 pounds superphosphate.....	884	156	900	185	895	150	837	138
Rock phosphate equivalent to 200 pounds superphosphate.....	819	143	832	203	874	177	864	153
Rock phosphate equivalent to 500 pounds superphosphate.....	929	138	780	177	973	164	764	164
Rock phosphate equivalent to 1,000 pounds superphosphate.....	962	156	874	148	942	161	822	193
Average.....	899	148	847	178	921	165	822	162

TABLE 5 — *Phosphoric acid content (per cent) of soybean hay grown on different types of soil under different phosphate treatments, 1923*

## SUPERPHOSPHATE SECTION

Phosphate treatment (pounds per acre)	Phosphoric acid content (per cent) of soybean hay grown—						
	With lime			Without lime			
	Colts Neck loam	Sassafras sandy loam	Portsmouth loam	Colts Neck loam	Sassafras sandy loam	Portsmouth loam	
No phosphate.....	0 668	0 645	0 526	0 642	0 682		0 531
100, superphosphate.....	658	665	632	653	668		603
200, superphosphate.....	655	685	582	725	673		611
500, superphosphate.....	682	721	621	668	718		655
1,000, superphosphate.....	705	736	640	707	760		658
Average .....	675	704	620	689	705		632

## RAW ROCK PHOSPHATE SECTION

No phosphate.....	0 668	0 670	0 642	0 648	0 658		0 575
Rock phosphate equivalent to 100 pounds superphosphate.....	650	658	611	650	666		619
Rock phosphate equivalent to 200 pounds superphosphate.....	642	611	606	679	674		630
Rock phosphate equivalent to 500 pounds superphosphate.....	686	684	617	749	710		614
Rock phosphate equivalent to 1,000 pounds superphosphate.....	668	676	622	715	668		637
Average.....	662	657	611	690	680		625

acid in the grain, and the total amount of phosphoric acid removed by the crop

It will be noted that there was a gradual increase in the yield as the fertilizer was increased. The 100-pound application of fertilizer gave only a slight increase in yield over the check and no increase in percentage of phosphoric acid. The 250-pound application gave a distinct increase in yield and also a distinct increase in percentage of phosphoric acid. The 500 and 1,000 pound applications likewise gave increases in yield and in percentage of phosphoric acid in the grain, the latter giving an increase over the check of nearly 40 per cent phosphoric acid. When 1,000 pounds of fertilizer were applied, there was about three times as much phosphoric acid removed in the grain as in that from the check plot

TABLE 9—*Phosphoric acid content of corn (grain) when grown with different quantities of a concentrated fertilizer in a field experiment, 1930*

Plot No	Fertilizer treatment	Yield of dry shelled corn	Phosphoric acid content	Total phosphoric acid removed from soil
	Pounds per acre	Pounds	Per cent	Pounds
110 "-----"	0	1,050	1.053	11.06
111 "-----"	100	1,067	1.047	11.17
112 "-----"	250	1,474	1.249	18.41
113 "-----"	500	1,544	1.370	21.15
114 "-----"	1,000	2,151	1.457	31.34

\* Check

#### THE EFFECT OF PHOSPHORIC ACID TREATMENT ON MIXED HERBAGE

Phosphoric acid was determined in samples of mixed herbage from plots that had received different fertilizer treatments. Dr H B Sprague of the department of agronomy, who has been conducting this work, furnished the samples for these determinations and has kindly allowed the writers to use the data. The soil on which the plots are located is Chester stony loam. The fertilizers were applied in early April, and the pasture was clipped every two weeks. The samples from the different plots were composites of the different cuttings throughout the season and therefore do not represent the seasonal influence on the crop.

The results are shown in Table 10. In every case the grass from the phosphate-treated plots showed a higher percentage of phosphoric acid than did that from plots without phosphate treatment. While it is not safe to draw definite conclusions from so limited a number of determinations, the results do show a distinct increase in the phosphoric acid content of the hay where superphosphate was used. Doctor Sprague is of the opinion that these differences may be due largely to differences in the vegetation which resulted from the fertilizer treatment. He found, for example, more clover on the phosphate-treated plots than on those without phosphate.

This is a phase of the question which must be considered when dealing with mixed herbage. Also the influence of the time of cutting must not be overlooked. Reference has already been made to these points.

TABLE 7.—*Phosphoric acid content (per cent) of barley grown on Colts Neck sandy loam under different phosphate treatments, 1923*

## SUPERPHOSPHATE SECTION

Phosphate treatment (pounds per acre)	Phosphoric acid content (per cent) of barley grown—			
	With lime		Without lime	
	Grain	Straw	Grain	Straw
No phosphate.....	0 895	0 187	0 843	0 135
100, superphosphate.....	928	182	879	148
200, superphosphate.....	861	156	910	156
500, superphosphate.....	913	167	871	154
1,000, superphosphate.....	890	153	879	130
Average.....	898	165	885	147

## RAW ROCK PHOSPHATE SECTION

No phosphate.....	0 900	0 146	0 842	0 143
100, superphosphate.....	923	180	861	130
200, superphosphate.....	903	174	887	143
500, superphosphate.....	918	172	920	128
1,000 superphosphate.....	882	156	910	198
Average.....	907	171	895	150

TABLE 8.—*Phosphoric acid content (per cent) of grain and hay of various crops grown with different fertilizer treatments in a field experiment*

Plot No	Fertilizer treatment	Phosphoric acid content (per cent) of crops when grown—					
		Without lime			With lime		
		Timothy hay, 1926	Oats grain, 1929	Wheat grain, 1930	Timothy hay, 1926	Oats grain, 1929	Wheat grain, 1930
1	No fertilizer.....	0 494	1 226	1 278	0 454	1 289	1 232
2	Muriate only.....	482	1 151	1 267	436	1 186	1 220
3	Superphosphate only.....	517	1 128	1 082	494	1 295	1 249
4	Minerals.....	505	1 128	1 130	540	1 283	1 284
9	Minerals and nitrate of soda.....	482	1 364	1 202	476	1 375	1 237

\* Minerals = 320 pounds of superphosphate and 160 pounds of muriate of potash an acre

PHOSPHORIC ACID CONTENT OF CORN (GRAIN) GROWN ON PLOTS RECEIVING DIFFERENT AMOUNTS OF A HIGH-ANALYSIS FERTILIZER IN FIELD EXPERIMENTS, 1930

The soil on which the corn used in the field experiment of 1930 was grown is a Sassafra loam of medium quality containing about 0.1 per cent phosphoric acid. It has been in corn continuously for several years, the respective plots receiving the same fertilizer treatment each year. The fertilizer analyzed 16 per cent ammonia, 18 per cent phosphoric acid, and 16 per cent potash. It was applied in the quantities indicated in Table 9. This table also reports the yield of dry shelled corn in pounds per acre, the percentage of phosphoric

Not enough work has been done on potatoes from these plots to make the work conclusive, but it indicates that on a soil moderately supplied with phosphoric acid, applications of superphosphate do not materially influence the phosphoric acid content of the potato.

In the early fall of 1930, rye was seeded on the potato plots referred to above without any further fertilizer treatment, and on November 1, samples of the young rye were collected for phosphoric acid determinations. The results on the dry material are shown in Table 12.

TABLE 12—*Phosphoric acid content (per cent) of young rye plants grown under different fertilizer treatments*

Fertilizer mixture			Phosphoric acid in young rye	Fertilizer mixture			Phosphoric acid in young rye
N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O		N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	
4	0	4	1.12	4	12	4	1.19
4	4	4	1.05	4	16	4	1.33
4	8	4	1.12				

When the 4-4-4 and 4-8-4 fertilizer mixtures were used for the potatoes there was no increase in the percentage of phosphoric acid in the rye, when the 4-12-4 fertilizer mixture was used there was a small increase; and when the 4-16-4 fertilizer mixture was used there was an increase of slightly more than 18 per cent in the phosphoric acid content. It would seem reasonable to conclude that heavy applications of phosphoric acid would affect pasture grasses in very much the same way as it affected the young rye.

### SUMMARY

Tables are given showing the phosphoric acid content of a number of crops grown in cylinders and also on field plots where different fertilizer materials have been used and where different amounts of superphosphate have been applied over a period of years—in one case over a period of 30 years.

So far as the work reported is concerned, light applications of superphosphate—100 to 250 pounds an acre—did not, in most cases, materially influence the phosphoric acid content of the crop. With heavier applications—500 to 1,000 pounds an acre—there was usually some increase in the phosphoric acid content of the dry matter. In some cases the increase was as much as 40 per cent.

In the case of potatoes, increasing the amount of superphosphate applied seemed to have no influence on the phosphoric acid content of the crop.

Phosphoric acid determinations were made on a limited number of samples of mixed herbage from plots with and without phosphate. The results indicate that the phosphate treatment tends to increase the percentage of phosphoric acid in the hay. However, attention is called to the fact that such increases may be due to changes in the type of vegetation which the phosphate treatment causes rather than actual increase in a specific plant.

TABLE 10—*The effects of phosphoric acid treatment and the application of other fertilizers upon the phosphoric acid content (per cent) of mixed herbage*

No.	Treatment <sup>a</sup>	Percentage of phosphoric acid as P <sub>2</sub> O <sub>5</sub>
2	Lime.....	0.756
7	Lime plus superphosphate.....	952
10	Lime plus muriate of potash.....	704
11	Lime plus muriate of potash plus superphosphate.....	998
12	Lime plus superphosphate plus muriate plus nitrate.....	958
13	None.....	721
18	Superphosphate.....	940
21	Muriate of potash.....	687
22	Superphosphate plus muriate of potash.....	940
23	Superphosphate plus muriate plus nitrate.....	940
	Average.....	
	(Superphosphate.....)	955
	(No superphosphate.....)	717

<sup>a</sup> Pounds per acre. Lime, 670, superphosphate, 600, muriate of potash, 100, and nitrate of soda, 100

### INFLUENCE OF PHOSPHORIC ACID TREATMENT ON THE PHOSPHORUS CONTENT OF POTATOES AND YOUNG RYE

The soil for the experiment on potatoes and young rye is a Sassafras loam of fair quality. It contains 0.11 per cent phosphoric acid. The work was started in 1924, and the plots have received annual applications of fertilizer made so that the minimum application has been 1,600 pounds an acre. The minimum application of 16 per cent superphosphate has been 400 pounds an acre and the maximum 1,600 pounds. The treatments for the different plots are shown in Table 11.

TABLE 11—*Treatment of plots used in potato and rye experiments*

Plots (number)	Fertilizer mixture			Pounds of phosphoric acid applied per acre	Plots (number)	Fertilizer mixture			Pounds of phosphoric acid applied per acre
	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O			N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	
<sup>a</sup> 4	4	0	4	0	2	4	12	4	102
2	4	4	4	64	2	4	16	1	256
2	4	8	4	128					

<sup>a</sup> Checks

Samples of potatoes from check plots and from those receiving the 4-16-4 fertilizer were analyzed for phosphoric acid in 1924 and in 1930. In 1924 the check plot gave 0.16 per cent phosphoric acid in the potatoes and the 4-16-4 plot also gave 0.16 per cent. In 1930 the check plot gave 0.14 per cent phosphoric acid in the potatoes, and the 4-16-4 plot 0.145 per cent.

Van Slyke<sup>6</sup> in his table of analyses of different crops gives 0.15 as the average percentage of phosphoric acid in potato tubers.

<sup>6</sup> VAN SLYKE, L. L. FERTILIZERS AND CROPS, OR, THE SCIENCE AND PRACTICE OF PLANT-FEEDING, A PRESENTATION OF FACTS, GIVING PRACTICAL METHODS FOR USING FERTILIZERS IN CROP-GROWING, WITH SPECIAL EMPHASIS ON THE REASONS UNDERLYING THEIR USE, AND ON THE CONDITIONS OF THEIR GREATEST EFFICIENCY. p. 719. New York. 1912.

# VITAMIN A AND PROTEIN CONTENT OF VARIOUS FISH MEALS<sup>1</sup>

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## INTRODUCTION

Although fish meals are derived from widely different sources of natural supply and are subject to very different methods of treatment before being placed on the market, they are usually classed by the stockman who employs them in animal feeding merely as fish meal. On the assumption that these products, differing in origin and in method of manufacture, might also differ in nutritive value, an investigation was undertaken to ascertain whether there are any important nutritive differences among the products which are popularly known under the general term "fish meal." This investigation has been in progress for three years, and the results so far obtained are here reported.

## DESCRIPTION OF FISH MEALS USED

During the course of the investigation seven different fish meals have been employed. For convenience of reference these products are listed in Table 1, with certain data as to their sources and the methods of their manufacture. White fish meal is made from non-oily fish, principally cod and haddock, and consists of the heads, tails, fins, backbones, and the flesh remaining from the cutting of the fillets. The entrails are removed at sea and thus are not included. The material is cooked and then dried. The three products used were all vacuum dried at low temperatures, as shown in the table. The menhaden is an oily fish, and the meal is the residue after the removal of a portion of the oil by pressing and cooking. The dried product still contains from 4 to 12 per cent of oil. The higher values are found in meals containing unpressed fish from catches too low in oil to warrant pressing. Most of the menhaden meal now on the market is flame dried at a high temperature, as were two of the products studied. The third product listed was a steam-dried meal produced experimentally by the Bureau of Fisheries, as described by Harrison (6).<sup>2</sup>

TABLE 1.—*Fish meals used during the investigation*

Meal	Parts of fish included	Method of drying
White, No. 1.....	Entire fish less side meat (fillets), less entrails.....	Vacuum dried under 105° F.
White, No. 2.....	.....do.....	Vacuum dried at about 116° F.
White, No. 3.....	.....do.....	Vacuum dried at about 100° F.
Menhaden, No. 1.....	Entire fish less a portion of the oil.....	Flame dried at 500°-600° F.
Menhaden, No. 2.....	.....do.....	Flame dried at 500°-600° F.
Menhaden, No. 3.....	.....do.....	Steam dried at 120°-200° F.
Pulchard.....	.....do.....	Indirect-heat dried at 300°-400° F.

<sup>1</sup> Received for publication Dec. 4, 1931, issued May, 1932.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 603.

Undoubtedly there may be conditions in which it would be profitable to apply rather large quantities of superphosphate for the purpose of increasing the phosphoric acid content of the dry matter.

The soils used in these experiments varied in phosphoric acid content from 0.1 to 0.8 per cent, but the phosphoric acid content of the soil evidently had little or no influence on the phosphoric acid content of the crop, since soybeans grown on Colts Neck loam containing 0.8 per cent  $P_2O_5$  contained no more phosphoric acid than did those grown on Sassafras loam with about 0.1 per cent  $P_2O_5$ .

In comparison with the changes that may be wrought in the nitrogen content of plants by the application of nitrogenous fertilizers, the changes in phosphorus content produced by phosphate treatments are relatively small.

TABLE 3—*Growth of male and female rats when fed various fish meals, or tankage and supplements, combined with corn*

Diet			Animals		Average weight at start		Average gain in 10 weeks	
No	Ingredients	Protein content	Males	Females	Males	Females	Males	Females
		<i>Per cent</i>	<i>Number</i>	<i>Number</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>
1	Corn and white meal No 1.....	14.4	5	5	53	58	215±8	128±4
2	Corn and white meal No 2.....	14.8	5	5	58	56	237±9	147±3
3	Corn and white meal No 3.....	14.6	5	5	62	46	217±8	135±5
4	Corn and menhaden meal.....	14.3	5	5	55	59	196±5	141±6
5	Corn and pilchard meal.....	14.3	5	5	57	53	153±6	118±7
6	Corn and tankage.....	14.5	5	5	53	56	177±8	117±4
7	Corn and tankage plus yeast and cod-liver oil.....	14.5	5	5	52	56	221±5	125±4
8	Corn and white meal No 1.....	11.5	5	5	56	49	153±6	119±6
9	Corn and tankage.....	11.5	5	5	52	55	102±7	94±4

It is noted in Table 3 that five male and five female rats were used with each diet. Because of their different growth rates the data for the two sexes are averaged separately. Since the males grew faster, their quantitative requirements for the various nutritive factors exceed those of females, and thus growth experiments with males constitute a more rigid test.

From a study of the data for average gain in 10 weeks on the first six diets, it is seen that in general the white meals gave better results than the other supplements. Considering odds of 30:1 as practical certainty, the results with the males show each cod and haddock meal significantly superior to tankage. Numerically superior results are shown in each comparison for the females also, and in the case of white meal No. 2 the difference is clearly significant. At the lower level of protein intake (diets Nos. 8 and 9) significantly better results are shown for white meal No. 1 in the case of both sexes. On the basis of the results with males each white meal proved significantly superior to pilchard meal. The results with females are numerically superior in the case of the white meals. Menhaden is shown to be superior to pilchard—numerically for females, significantly for males. White meal No. 2 gave significantly better results than menhaden meal in the case of the males, and numerically so in the comparison with females. The results with diets Nos. 6 and 7 show that at least one of the limiting factors in the corn and tankage diet was its vitamin content.

The results set forth in Table 3 clearly indicated that marked nutritive differences exist among certain of the products studied but furnished no definite information as to their nature. Therefore, more specific studies were undertaken.

#### VITAMIN A STUDIES

It was recognized that differences between oily and nonoily fish and differences as regards heat treatment in drying might be expected to result in meals varying markedly in vitamin A content. It was decided to study this question by a comparison of the vacuum-dried white meal with the menhaden meal dried at a much higher temperature. The analyses of the meals used in these and succeeding studies are given in Table 4.

Pilchard meal consists of whole sardines from which a portion of the oil may have been extracted, and of heads, tails, and entrails resulting as a by-product from the canning of sardines. In analysis the product is similar to menhaden meal.

The drying temperatures shown in Table 1 are based upon information from the manufacturers. Some of the products were undoubtedly subjected to higher initial temperatures than those shown. No information is available as to the time factors involved. For a detailed discussion of the methods of preparation of various fish meals the reader is referred to the reports by Harrison (6) and Fiedler (5).

### PRELIMINARY GROWTH STUDIES

Two preliminary experiments were carried out in 1928-29, which were expected to give general information only, as a basis for planning more specific studies. These experiments are reported briefly with summarized results.

First a study was made in which a diet of 92 parts of yellow corn and 8 parts of white meal No. 1 was compared with a combination of 90 parts of corn and 10 parts of tankage. Both diets contained approximately 13.5 per cent of protein. Twelve male rats were placed upon each diet shortly after being weaned, and growth records were kept for 16 weeks. The data are presented in a condensed form in Table 2. They indicate that the diet containing fish meal was superior to the tankage combination. This result is in accord with the observations of other investigators, as recently reviewed by Manning (10).

TABLE 2—*Growth of 12 male rats on a diet of corn and fish meal as compared with growth of 12 male rats on a diet of corn and tankage*

Diet	Average weight of rats at start	Average gain in weight during 6 weeks	Average gain in weight during 16 weeks
Corn and white meal No. 2.....	Grams 46	Grams 129±3	Grams 229±5
Corn and tankage.....	47	86±5	160±6

A survey experiment was next carried out in which six different fish meals were studied. The essential data are shown in Table 3. In the first five diets corn and fish meal were combined in the proportion 90 to 10. In diet No. 6, 89 parts of corn to 11 of tankage were used. The six diets are similar in protein content, as shown by the percentage figures which are based on an analysis of the fish meals used and the average figure of 9.3 per cent for corn. Diet No. 7 is the same corn and tankage combination used in diet No. 6 plus 0.5 of yeast and 3 drops of cod-liver oil daily per rat. These additions were made in view of the poorer results obtained with tankage in the first experiment to ascertain whether they were due to vitamin deficiencies. Diets Nos. 8 and 9 were used to compare the two combinations at a lower protein level.

liberal and in nearly the same quantities, indicating that failure to eat was not the primary cause of the poorer growth on diets Nos. 11 and 12. During the seventh week, despite their very slow growth, the rats on diets Nos. 11 and 12 were eating 15 to 25 per cent more food per unit of body weight than those on diet No. 10, quantities markedly in excess of their maintenance requirements.

This vitamin A experiment was repeated, the same diets, Nos. 10 and 12, being used, as shown in Table 5, and also diet, No. 13, containing the steam-dried menhaden meal. Ten rats were given each ration. The growth data are shown in Figure 1, B. Again the rats on the white-meal diet grew practically normally during the experimental period and no xerophthalmia developed, a marked contrast to the performance on the menhaden diets. Apparently the rats used in this second experiment had less reserve vitamin A in their bodies

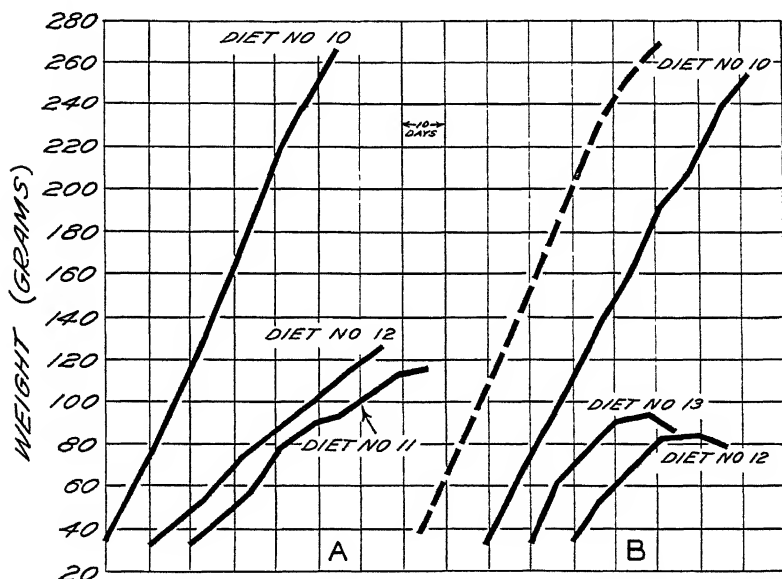


FIGURE 1—Growth of rats during the first (A) and second (B) experiments carried on to determine the vitamin A content of fish meals. The broken line represents normal growth in the colony. See Table 5 for composition of rations.

at the start, for the xerophthalmia developed earlier on diet 12 than it did in the first experiment. At the close of the fifth week all the rats on diets Nos. 12 and 13 exhibited the disease, and all except two (on diet No. 12) were losing weight. Thus these groups were discontinued at this time. It is seen in the chart that the rats on the steam-dried menhaden meal (diet No. 13) grew no better than those on the flame-dried product (diet No. 12). At the close of the five weeks all of them contracted xerophthalmia and all were losing weight.

In order to make certain that failure to consume as much of the menhaden meals was not responsible for the poor results with them, a further comparison of the white meal and the steam-dried menhaden was made by the curative method. After the growth of the experimental animals had ceased upon a vitamin-A-free diet the meals were added at the rate of 1 g per rat per day. The results of this test are

TABLE 4—Percentage composition of white and menhaden fish meals

Meal	Water	Ash	Protein	Fat
White, No 1.....	9 7	19 01	64 08	2 60
Menhaden, No 1.....	5 49	21 60	59 56	7 21
Menhaden, No 2.....	5 19	21 03	60 82	6 14
Menhaden, No 3.....	7 00	16 50	59 68	11 83

In the first experiment the white meal and menhaden meals Nos 1 and 2 were used. They were fed as diets Nos 10, 11, and 12, shown in Table 5. It is noted that the diets were made up in such a way as to provide substantially the same percentage of protein and the same calorific value in each. Dried yeast was added to provide the vitamin B complex. Irradiated ergosterol was fed separately to provide vitamin D.

TABLE 5—Composition of diets used in vitamin A experiments

Ingredients	Composition of diet No —			
	10	11	12	13
White meal No 1.....parts.....	19 0			
Menhaden meal No 1.....do.....		20		
Menhaden meal No 2.....do.....			20	
Menhaden meal No 3.....do.....				20
Lard.....do.....	20 5	20	20	19
Sugar.....do.....	10	10	10	10
Cooked starch.....do.....	45 5	45	45	46
Yeast.....do.....	5	5	5	5
Protein.....per cent.....	14 38	14 51	14 77	14 53

\* In addition to the above each rat received 0 001 mg of irradiated ergosterol daily

Ten male rats were placed on each diet shortly after being weaned and were fed for a period of eight weeks. The growth data are shown in Figure 1, A. The broken curve represents the normal growth of the colony. The experimental growth curves are plotted from the average weekly weights of the 10 rats. It is noted that the rats on the white meal made practically normal growth. No signs of xerophthalmia developed in the 8-week experimental period. On the other hand, the growth on the other two diets became progressively poorer as the experiment continued, and the total growth reached at the end of eight weeks was less than half that produced on diet No. 10. On diet No. 11 the first case of xerophthalmia appeared during the sixth week. During the next week the trouble developed in seven other rats, and the total cases reached eight during the last week. None of these eight rats made any gain during this last week, and some of them lost weight. The small increase for the group as shown in Figure 1, A, was due entirely to the two rats which thus far showed no signs of xerophthalmia. The history of the development of this eye trouble was similar with diet No. 12. At the close of the experiment only one rat failed to show symptoms of the disease, and two had already died from it.

The rats were allowed to feed ad libitum, but food-intake records were kept during the first two weeks and again during the seventh week. During the first two weeks all the diets were consumed in

diets containing these meals. The diets were made up to supply a level of 7 per cent of protein calories. These diets are shown in Table 6. The experiment lasted nine weeks, and the growth records are presented in Figure 3. It is noted in Figure 3 that the best growth was made on the white meal (diet No. 17) and the poorest on the flame-dried menhaden (diet No. 18), the steam-dried product (diet No. 19) occupying an intermediate position.

TABLE 6.—*Composition of diets fed in protein experiment in which the group-feeding method was used*

Ingredients	Composition of diet No.—		
	17	18	19
White meal.....parts.....	12.9		
Flame-dried menhaden meal.....do.....		13.8	
Steam-dried menhaden meal.....do.....			13.5
Lard.....do.....	19.7	19.2	18.5
Sugar.....do.....	10.0	10.0	10.0
Cooked starch.....do.....	55.4	55.0	56.0
Salt mixture.....do.....	2.0	2.0	2.0
Protein.....per cent.....	8.3	8.2	8.1
Energy per gram.....calories.....	4.74	4.69	4.74
Protein calories.....per cent.....	7.0	6.9	7.0

\* In addition to the above each rat received 200 mg of yeast and 3 drops of cod-liver oil daily.

These results were considered as suggestive only, and a second experiment was conducted in which individual food records were kept. The rations used are shown in Table 7. In this experiment a higher protein level was used than in the first experiment; also more starch and less lard were included.

TABLE 7.—*Composition of diets fed in protein experiment in which the limited-individual feeding method was used*

Ingredients	Composition of diet No.—		
	20	21	23
White meal.....parts.....	14.7		
Flame-dried menhaden meal.....do.....		15.3	
Steam-dried menhaden meal.....do.....			15.6
Lard.....do.....	15.5	14.9	14.0
Sugar.....do.....	10.0	10.0	10.0
Cooked starch.....do.....	58.8	58.8	59.4
Salt mixture.....do.....	1.0	1.0	1.0
Protein.....per cent.....	9.3	9.3	9.3
Energy per gram.....calories.....	4.42	4.41	4.45
Protein calories.....per cent.....	8.6	8.6	8.6

\* In addition to the above each rat received 200 mg of yeast and 3 drops of cod-liver oil daily.

The second experiment was conducted in accordance with a modification of the paired-feeding method. The available animals were divided into groups of three, the animals comprising a given trio, being as nearly alike as possible. Six groups were selected in this way. One animal from each trio was placed on each of the three rations. For a given trio the food intake was governed by the animal consuming the least, but as a given animal attained a greater weight than the animal regulating the food intake of the group, it was fed additional rations to enable it to meet its higher maintenance require-

shown in Figure 2. It is shown that the white fish meal caused a resumption of growth nearly comparable to that of the butter, while the steam-dried menhaden was ineffective, and that the animals continued to lose weight and died as did the controls. With the white meal the xerophthalmia was cured, while with the menhaden it became progressively worse until death.

It is clear from these three experiments that the vacuum-dried white meal proved markedly superior to the flame-dried and steam-dried menhaden meals in vitamin A content. It was rather surprising to find the white meal so effective in view of its low oil content, and of the further fact that the oil present is presumably a body oil. Haddock oil, which probably makes up a considerable proportion of the oil present, is certainly not rich in vitamin A, according

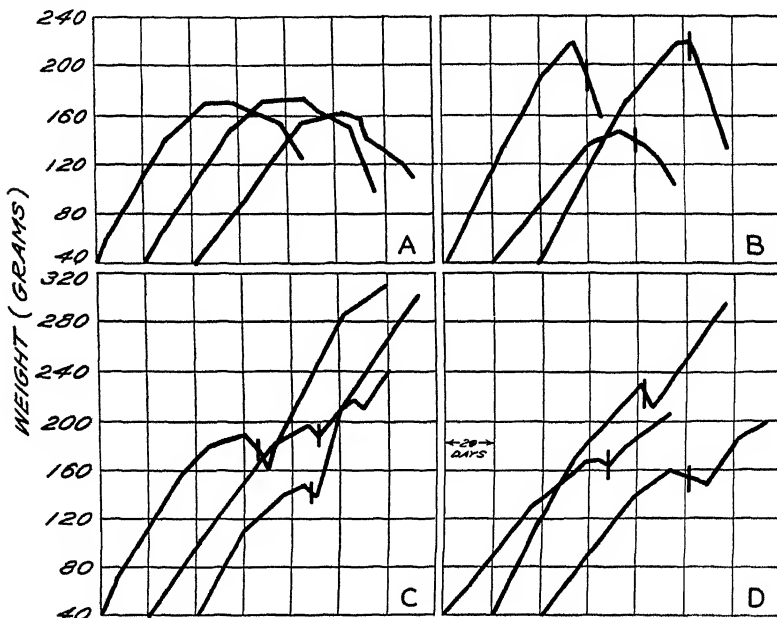


FIGURE 2—Growth of rats when fed butter and fish meals as a source of vitamin A. A, Control, B, menhaden meal, C, butter, D, white meal. Vertical lines indicate points at which supplements were added to the basic ration.

to the work of Kik and McCollum (9). Bohstedt and coworkers (3) have shown menhaden meal, presumably a flame-dried product, to be lacking in vitamin A. Whether the poor results obtained with the menhaden products were due to the destructive action of the drying process or to a low vitamin A content in the raw material is not brought out by these experiments. Earlier experiments by Maynard and Miller (11) showed that menhaden oil contains vitamin A in limited amounts.

#### PROTEIN STUDIES

A study of the nutritive value of the proteins of representative fish meals was next undertaken. White meal No. 1 and menhaden meals Nos. 2 and 3 were used. The first experiment consisted of a comparison of groups of nine rats each given access at all times to

ences in maintenance requirements as would provide each pair with an equal food intake for growth. In order to do this it was obviously necessary to have values for the maintenance requirements of rats of different weights. The values used by the writers were worked out from data by Osborne and Mendel (14). These investigators present the intakes of a purified diet by rats of different weights receiving just enough food to hold them at nearly constant weight for periods of three weeks. From their data it was possible to calculate the calories required per week per gram of rat for maintenance. On the basis of the caloric value of 4.45 (the highest one shown in Table 7), it was calculated that the following quantities of the diets used in the experiments here described would be required for maintenance at the different weights:

Weight of rat in grams	Grams of diet required for main- tenance per gram of rat per week
50-75.....	0.50
75-100.....	.46
100-125.....	.36
125-150.....	.36
150-175.....	.35

It is recognized that the calculation of these values involved certain assumptions. There is an experiment in progress in this laboratory by McCay and Crowell which is furnishing very complete data regarding the maintenance requirements of rats. On the basis of calculations from the data available to date, namely, for rats of various weights up to 100 g, the requirements have been found to be approximately 10 to 15 per cent less than those represented by the data in the preceding table. The results of this study now in progress suggests that the maintenance allowances used in these experiments were somewhat too high. The bearing of this upon the results of the experimentation will be referred to later.

During the first two weeks of this experiment each rat of a given trio was fed the same quantity of food, governed by the consumption of the animal eating the least. At the close of this period, in each trio the rat that was consuming the least weighed markedly less than the other two. From this time on the two heavier rats of the trio were given additional food to provide for their extra maintenance needs. For example, rat 19 of trio 1 (Table 8) weighed 64 g at the end of the two weeks, while rat 18 weighed 80 g and rat 32 weighed 86 g. Since rat 18 was 16 g heavier than rat 19, it required  $16 \times 0.46$ , or 7.4 g additional food a week for maintenance, on the basis of the requirement for rats weighing 75-100 g previously listed. Similarly, rat 32 required 10.1 g additional. During the following week these amounts were allowed the rats in question in addition to the amount consumed by rat 19. At the close of the third week similar calculations were made, and so on for each trio to the close of the experiment.

The food intakes and the growth records are presented in Table 8. These records cover an experimental period of nine weeks. In trio 6 the rat on the steam-dried menhaden died from an accident during the fourth week, and thus the records are presented for the other two only. In comparing the gains made it is seen that in all six comparisons the rat receiving the white meal made a larger gain than its mate on flame-dried menhaden meal. An analysis of these

ment This procedure, which differs from the absolute equalization of food used by Mitchell in his paired-feeding experiments, requires explanation

The argument for the absolute equalization of food in the paired-feeding method is that if one is superior to another for growth its superiority should be evident at equal levels of food intake. However, as the animal on the superior ration increases in weight over its mate its maintenance requirement becomes greater than that of its mate Under these conditions an equal food intake for both means that the larger animal must be using a larger proportion for maintenance, and less remains for growth promotion Under these conditions an absolute equality of food intake means that the quantities available for the specific function which is being used as the criterion in comparing the two rations are not equal The faster-growing animal is penalized It may be accepted that if a given ration continues to produce superior growth under these conditions, the conclusion that it is superior is made stronger thereby

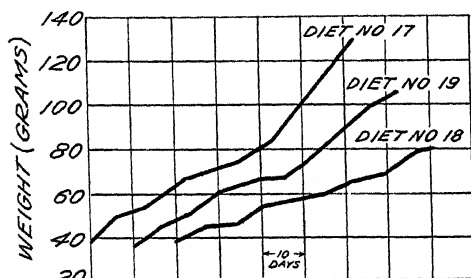


FIGURE 3—Growth of rats when allowed unrestricted access to fish meals as a source of protein, at a level of 7 per cent of protein calories See Table 6 for composition of rations

efficiency of various proteins in inducing growth, keeping the food intakes the same for the different rations In their discussion they state (15, p 4):

\* \* \* inasmuch as the animals were all receiving the same absolute amounts of food on the corresponding day of each period, the rapidly growing animals were actually put at a disadvantage in having a smaller allowance of food per unit of body weight \* \* \*

Armsby (1), in his plan for studies of the growth of calves by means of paired-feeding experiments, specified that the ration should be adjusted in accordance with the increasing live weight of the animal, but that the energy supply of both animals of a pair should be kept the same per 1,000 pounds of live weight In discussing the paired-feeding method, Mitchell and Beadles (12, p 227) state:

If one ration is superior to another in the support of an animal function such as growth, its superiority should be evident when the intakes of both rations by comparable animals is the same, either absolutely or in proportion to some determinant of food requirements, such as body weight, or a mathematical function of body weight

In comparing rations for milk production it is standard practice to provide for the different maintenance requirements of the animals first and then to equalize the intakes in terms of the function being studied

In view of these various considerations it seemed worth while to try out a procedure which would involve such adjustments for differ-

pair giving contrary results the difference in favor of the flame-dried product was only 6 g. An analysis of the data for the five pairs by Student's method reveals odds of 40:1 favoring the steam-dried meal. It seems highly probable, therefore, that the results demonstrate its superiority, despite the erratic results from the comparison in trio 5.

For each trio the food-intake data are divided into a basal intake, which was governed by the amount eaten by the animal consuming the least, and an extra allowance for maintenance, which was given the faster-growing rats, based on their increased weight as previously described. Assuming that the extra allowances for maintenance were actually needed for this purpose, the gains per gram of basal food could be considered quantitative measures of the relative efficiencies of the different meals as sources of protein for growth. However, as has been stated, experiments in progress indicate that the maintenance allowances were somewhat excessive. This would serve to give the faster-growing and thus heavier animals an advantage as regards food available for growth. Thus the differences in protein efficiency can not be considered as large as the differences in gain per unit of basal food would suggest. As a further measure, the gain per gram of total food is shown. It is noted that without exception the white meal proved superior to both the menhaden meals in this respect and that the steam-dried menhaden proved superior to the flame-dried in four out of five cases and equal in the other.

The method here used of allowing additional food in accordance with higher maintenance needs worked out satisfactorily in the present instance and is believed to be worthy of further trial. It seems theoretically sound, provided the experimenter has data as to maintenance requirements applicable to the conditions in his colony. Perhaps some of the published data on the basal metabolism of the rat could be adapted for the purpose. Whatever data are used, it is evident from the studies of Benedict and MacLeod (2) on the heat production of the rat that account must be taken of environmental temperature.

As a result of the trial here reported, certain possible difficulties in carrying out the method can be foreseen. The use of trios instead of pairs may present certain difficulties even where the food intakes are absolutely equalized, and it is believed that the use of the present method should be limited to pairs. Even here one can foresee the possibility that the rat consuming the least might be the heavier if its ration was much more efficient but much less palatable. Under these conditions, with the basal intake being governed by the heavier animal, an additional amount for maintenance figured for the latter would of course not be consumed. The only practical way would be to decrease the food given the lighter by the amount in question. Again with the usual situation of the lighter rat consuming the least, the appetite of its mate might not at all times prove sufficiently greater to cause it to consume completely its additional allowance for maintenance. Even if either of these possibilities happened only in an occasional week, it would serve to complicate the working out of the method. None of these possible complications were encountered in the present experiment. Further trials of it are needed to show whether they are likely to happen with sufficient frequency to make the method impracticable.

data by Student's methods shows that the odds favoring the white meal are greater than 3,000:1. The white meal is also shown superior to the steam-dried menhaden in the five comparisons made. A statistical analysis shows that the odds are 356:1 in favor of the white meal.

TABLE 8—*Growth and food records (grams) of rats during a 9-week protein experiment in which the limited-individual-feeding method was used*

Item	Growth and food consumption data for rats in—								
	Trio 1, rat No —			Trio 2, rat No —			Trio 3, rat No —		
	19 on flame-dried menhaden meal	18 on steam-dried menhaden meal	32 on white fish meal	30 on flame-dried menhaden meal	31 on steam-dried menhaden meal	28 on white fish meal	275 on flame-dried menhaden meal	276 on steam-dried menhaden meal	277 on white fish meal
Initial weight .....	39	41	39	44	47	45	39	37	35
Final weight .....	112	161	178	123	159	215	92	156	180
Gain .....	73	120	139	79	112	170	53	119	145
Basal food intake .....	414	417	413	477	477	480	380	382	384
Extra food for maintenance ..	0	88	104	0	40	93	0	89	129
Total .....	414	505	517	477	517	573	380	471	513
Gain per gram of basal food ..	18	29	34	17	24	35	14	31	38
Gain per gram of total food ..	18	24	27	17	22	30	14	25	28

Item	Growth and food consumption data for rats in—								
	Trio 4, rat No —			Trio 5, rat No —			Trio 6, rat No —		
	10 on flame-dried menhaden meal	16 on steam-dried menhaden meal	11 on white fish meal	24 on flame-dried menhaden meal	25 on steam-dried menhaden meal	26 on white fish meal	12 on flame-dried menhaden meal	13 on steam-dried menhaden meal	17 on white fish meal
Initial weight .....	36	40	41	34	32	34	35	(a)	31
Final weight .....	111	144	182	116	108	154	93	(a)	156
Gain .....	75	104	141	82	76	120	58	(a)	125
Basal food intake .....	431	431	431	413	411	414	388	—	389
Extra food for maintenance ..	0	66	119	33	0	96	0	—	81
Total .....	431	497	550	446	411	510	388	—	470
Gain per gram of basal food ..	17	24	33	20	18	29	15	—	32
Gain per gram of total food ..	17	21	26	18	18	21	15	—	27

<sup>a</sup> Died accidentally.

A comparison of the data for steamed menhaden with those for flame-dried menhaden shows that greater gains were made on the former in four of the five comparisons. It is sometimes considered that with five pairs the results with every pair should favor a given ration if they are to be considered significant in demonstrating the superiority of the ration. However, such a criterion does not take full advantage of the data in the present comparison. In the four pairs in which the steam-dried meal proved superior the differences in gains range from 29 to 66 g, the mean being 44 g, while in the one

menhaden and the latter superior to a flame-dried menhaden as regards protein efficiency for growth. The results suggest that differences in heat treatment are at least partially responsible for the nutritive differences found.

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This second protein experiment checks the first one in showing that the protein of the vacuum-dried white fish meal is superior to that of the menhaden meals. It seems probable that the differences in heat treatment are primarily responsible for these differences. In fact, since the menhaden meal contains the entire fish, including the entrails, while the white meal consists of residues from the cutting of fillets and does not contain any entrails, one would rather expect any difference in the raw material to be in favor of the menhaden. On the other hand, Ingvaldsen (7) has shown in his nitrogen-partition studies that high temperatures have a detrimental effect upon protein quality. This investigator found that temperatures above 195° C. reduce certain essential amino acids, and he concluded that the biological value must be lessened thereby. Morgan (13) has reported that the protein of cereals subjected to dry heat or toasting at approximately 200° for 45 minutes is not well utilized for growth. The differences in heat treatment shown in Table 1 may be sufficient to explain the differences in protein efficiency found for the products studied in this experiment. Ingvaldsen (8) found that putrefaction also had a deleterious influence, a fact which may be a further explanation of the poorer results obtained with the flame-dried menhaden. It is recognized that putrefaction occurs to a certain extent in the course of the handling of the material before it is dried, in the case of some of the commercial products at least.

Evidence that the heat treatment is the primary factor concerned in the differences in the protein efficiency of fish meals is furnished by Daniel and McCollum (4). From a comparison of various fish meals on the basis of protein content, it is reported that vacuum-dried meals are superior to flame-dried meals and that vacuum-dried cod and menhaden meals are similar in feeding quality. Another finding of these investigators, which, however, is not in agreement with the results here reported, is that a steam-dried menhaden meal is equal or superior to a vacuum-dried white fish meal. On the basis of the analytical figures given, it is evident that a different white meal from the one employed in these experiments was used. It is possible that marked differences exist in the vacuum-dried white products on the market. From a practical standpoint further carefully controlled comparisons of the various commercial vacuum-dried products are needed, not only to ascertain whether there are marked differences among them, but also to ascertain how uniform the product of a given manufacturer is.

A continuation of the protein studies in this laboratory, by the nitrogen-balance method, has resulted in more specific information, which is reported by Schneider (16).

#### SUMMARY

Seven different fish meals, five of them commercial products sold for animal feeding, have been studied in growth experiments with rats. A preliminary experiment revealed marked differences among certain of the commercial products when fed as supplements to cornmeal, although all except one gave better results than tankage. Vitamin A experiments showed a vacuum-dried white fish meal to be a good source of this vitamin, in contrast to a steam-dried and to flame-dried menhaden meals which proved to be lacking in it. The vacuum-dried white meal was found to be superior to the steam-dried

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## COMPARATIVE PATHOLOGICAL HISTOLOGY OF THREE BACTERIAL DISEASES OF BEAN<sup>1</sup>

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### INTRODUCTION

With the description of *Bacterium flaccumfaciens* Hedges (6)<sup>3</sup> and of *Bact medicaginis* var *phaseolicola* Burk (1) in 1926, and Burkholder's comparative study (2) of the bacterial diseases of beans in 1930, several new bacterial maladies of the bean (*Phaseolus vulgaris* L.) were differentiated from the complex known as blight and believed to be caused by *Bact phaseoli*. E. F. Smith alone Hedges (6) presented a study of the cultural characteristics of *Bact flaccumfaciens* as compared with those of *Bact phaseoli*, while Burkholder differentiated these and other bean organisms on the basis of symptomatology, host range, and etiology.

In a previous paper the writer (17) reported investigations of bacterial blight of beans caused by *Bacterium phaseoli*, which dealt principally with the relation of the parasite to the host. It is the purpose of this paper to review briefly the seedling symptoms produced by the three blight organisms, and to compare particularly their pathological relation to the tissues in the seedling stage.

### SYMPTOMS

Since the symptoms of the bacterial blights were fully described by Burkholder (2), only a brief résumé of those on the seed and the seedling will be recorded here.

The symptoms produced by the three bacterial pathogens on the seed are difficult if not impossible to differentiate. In the case of vascular invasion they produce a yellow discoloration on light-seeded varieties. Even though *Bacterium medicaginis* var. *phaseolicola* is nonchromogenic in culture, Burkholder states that on white seeds the affected areas are characterized by a maize-yellow to cream color. With severe infection, the three organisms may cause shriveling of the seed, particularly if the infection takes place previous to the maturity of the seeds. On the other hand, it is more difficult to separate these diseases on dark-seeded varieties, especially when the infection is mild. Often bacterial exudate may be seen at the hilar region, and by examining this it is possible to distinguish *Bact phaseoli* and *Bact. flaccumfaciens* from *Bact medicaginis* var *phaseolicola*, but not from each other, since the color of the exudate of the first two organisms mentioned is yellow while that of the last is a grayish-white to cream color.

<sup>1</sup> Received for publication Aug. 31, 1931, issued May, 1932.

<sup>2</sup> The writer is indebted to L. L. Harter and W. A. Whitney for suggestions and criticisms in the preparation of the manuscript.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 632.

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in the lesion. When infection is very severe the plant topples over at the invaded region and death results.

Lesions are frequently found below the ground level. They first appear as water-soaked areas, later becoming red in color, and can be seen at the point where the young secondary roots emerge from the cortex. The lesion may extend upward, involving a considerable portion of the lowermost part of the hypocotyl.

The symptoms produced by *Bacterium flaccumfaciens* may be manifest when the seedlings are very small. According to Hedges (6, p. 2), seedlings—

not more than 2 or 3 inches high may be attacked. The wilting and shrivelling of the leaves is sometimes accompanied by a dull green or brownish green discoloration, and the whole plant may be dead before it has developed more than the first pair of leaves.

In many instances the first symptoms appear on the leaves as water-soaked spots which dry out very readily, become papery white in color, and are surrounded by a narrow water-soaked margin. The adjacent tissues are slightly drawn together because of the lack of development of the invaded area. The pulvinus of the leaf and petiole often becomes swollen, and small droplets of bacterial exudate accompanied by a cracking of the petiole may be seen. When the pulvini become infected the leaflets may droop.

#### MATERIALS AND METHODS

The plants used in the course of the investigation were grown in the greenhouse. Bean seeds of each lot infected with one of the three bacterial organisms were planted in sterile white quartz sand, and the pots subirrigated by placing them in a large pan of water. This method prevented possible secondary spread of the organisms, which might have taken place if the plants had been watered in the usual manner.

When lesions appeared on the seedlings, cultures were made from a portion of the plant to determine the specific organism present; the remaining portion was fixed in formal acetic alcohol, embedded in paraffin, and sectioned. Giemsa stain (with a 2 per cent aqueous Licht Grün or orange G as a counterstain) was employed for material invaded by *Bacterium phaseoli* and *Bact. medicaginis* var. *phaseolicola*. Material invaded by *Bact. flaccumfaciens* was stained with the Gram stain because of its positive reaction to this stain, which differentiated the bacteria clearly from the host tissues stained with orange G.

By the use of these differential stains, *Bacterium flaccumfaciens* was easily distinguished in the host from the other two organisms. *Bact. flaccumfaciens* did not stain clearly with the Giemsa stain, but stained very well with the Gram stain, whereas *Bact. phaseoli* and *Bact. medicaginis* var. *phaseolicola* were not clearly defined with the latter, but were distinctly stained in the host with the former. It was impossible to differentiate the latter two organisms by means of a staining reaction.

In the following discussion the migration of the three parasites—*Bacterium phaseoli*, *Bact. medicaginis* var. *phaseolicola*, and *Bact. flaccumfaciens*—will be traced from their penetration into the seed to their passage throughout the tissues of the seedling. Even though *Bact. phaseoli* and *Bact. medicaginis* var. *phaseolicola* produce quite

The symptoms produced on seedlings by *Bacterium phaseoli* and *Bact. medicaginis* var *phaseolicola* are more difficult to differentiate than those caused by *Bact. flaccumfaciens*, and hence they will be discussed together, except in those cases where the symptoms can be distinguished from one another.

When seeds invaded by either *Bacterium phaseoli* or *Bact. medicaginis* var *phaseolicola* are planted, only those slightly infected will germinate, those severely affected disintegrating in the soil. It is often difficult to detect symptoms in seedlings grown in the greenhouse before the end of about two to three weeks after planting, or until they have attained a height of 9 to 12 inches, but occasionally small water-soaked spots may be seen on infected cotyledons earlier.

The first macroscopic symptoms usually appear as angular, water-soaked spots conspicuous on the under sides of the primary leaves. These areas usually occupy similar positions on the two leaves, indicating that the infection took place while they were still folded between the cotyledons. These water-soaked regions can generally be distinguished from those produced by stomatal or secondary invasion, since the latter begin as small circular spots which upon enlargement may become angular. A yellow discoloration appears on the upper side of the leaf directly over the lesions. In this stage it is often possible to differentiate the symptoms produced by *Bacterium medicaginis* var *phaseolicola* and *Bact. phaseoli*, for the former produces a characteristic halolike zone, water-soaked at the center, which may vary from one-half to 1 inch in diameter. *Bact. phaseoli* also produces a small water-soaked spot which is surrounded by a deep-yellow border more regular in outline than that produced by *Bact. medicaginis* var *phaseolicola*.

Another symptom in the early stage of growth is characterized by a weakness of the pulvinus of the petiole or leaf, resulting in a drooping of the leaf during midday and a return to normal turgidity at night. This condition may continue for a few days, after which the invaded pulvinus becomes so weakened that permanent wilting takes place, subsequently resulting in the death of the affected parts. Only a single petiole or leaf may be so affected, the others remaining healthy. The invaded pulvinus may take on a reddish coloration, which extends along the petiole as a dark water-soaked region, accompanied by a longitudinal cracking of the tissues in which may be found a bacterial slime or exudate. This reddish discoloration in many instances follows for a short distance the main veins of the leaf. The bacteria invading the xylem vessels of the leaf may break out from these tissues at different points, causing the leaf to become somewhat puckered.

The lesions at the cotyledonary node may not appear for some time, but gradually they become dark and take on a water-soaked appearance. Later they turn a reddish brown, and at about this time the stem is so weakened at this point that the plant may break over, particularly when conditions of high humidity prevail. Under conditions of low humidity the symptom may not appear until about the time of pod formation, when the stem is girdled by the bacteria and from the weight of the top breaks off at the infected node.

Bacteria, which enter the cauline stomata or break out from invaded xylem vessels, may produce a longitudinal cracking of the stem, which later takes on a brick-red color. Often a bacterial slime may be seen

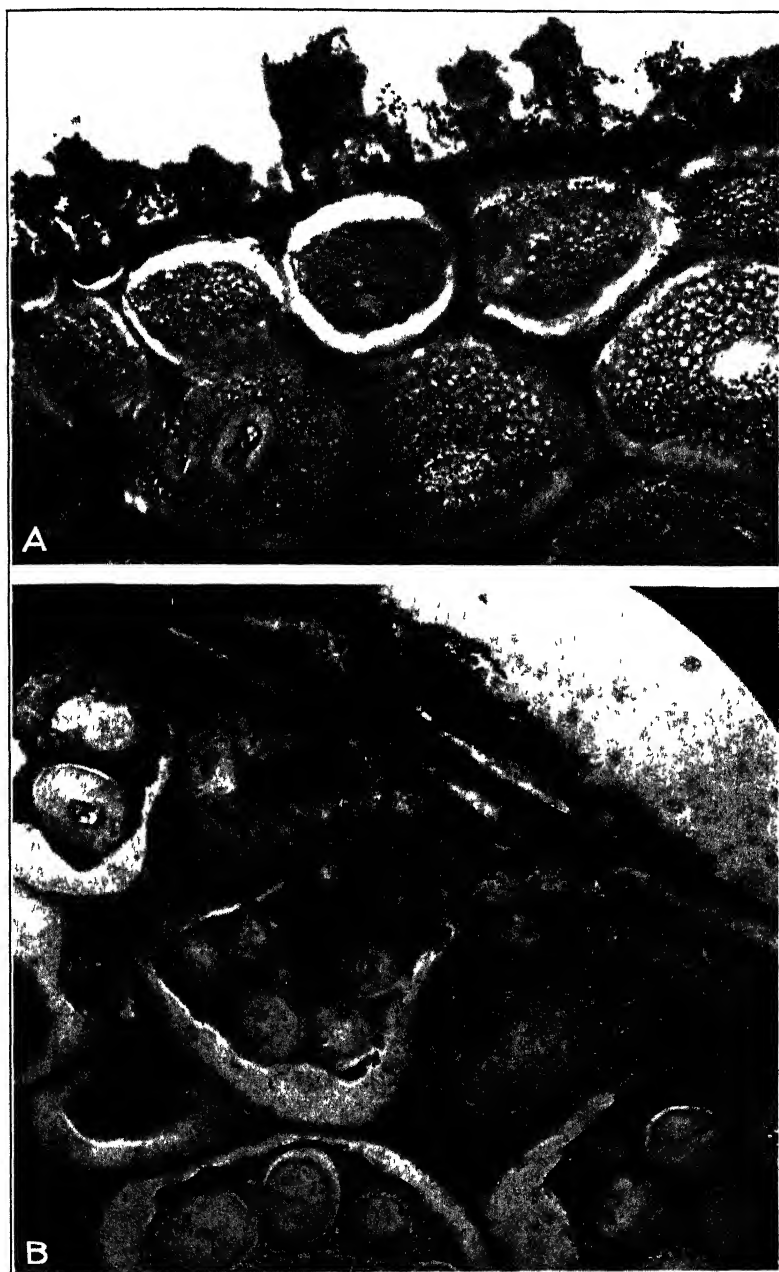


FIGURE 1—Invasion of bean cotyledon by *Bacterium phaseoli*. A, Bacteria entering small rifts in the epidermis and migrating throughout the tissues by way of the intercellular spaces. B, advanced stage of A, showing large masses of bacteria causing a decided enlargement of the intercellular spaces with a distortion of the adjacent cells  $\times 650$

similar effects on the host, each will be treated separately. With respect to cell-wall destruction the three organisms will be discussed as one, since all appear to cause a similar effect on the cells of the host.

#### RELATION OF BACTERIUM PHASEOLI TO THE HOST TISSUES INVASION OF THE SEED

Previous investigations by the writer (16) have shown that *Bacterium phaseoli* may enter the seed either through the vascular elements by way of the funiculus and raphe or through the micropyle, a natural opening in the seed. Entrance through the micropyle is made possible by the bacteria breaking out from the invaded funiculus, from the vessels of the dorsal suture of the pod, or from the parenchyma tissues of the pod where invasion began from stomatal penetration. It was also shown that the bacteria do not enter the cotyledonary tissues until germination, when by the imbibition of water the seed swells, often resulting in the pulling apart of many of the epidermal cells. The bacteria massed on the exterior of the cotyledons at this time may enter these rifts (fig 1, A and B), pass into the intercellular spaces of the adjacent cells, and in many cases cause the spaces to swell to enormous size with a distortion of the adjacent cells (Fig 1, B). They may then pass into the vascular elements and from there enter the xylem cells of the hypocotyl and epicotyl at the cotyledonary node. The organism may also enter the stem through the parenchyma tissue which connects the cotyledon with the stem. When slight infection occurs at the distal portion of the cotyledon, the bacteria may not traverse the tissues rapidly enough to enter the stem before the formation of the abscission layer. In such instances the young plant usually develops without becoming infected. On the other hand, cotyledonary invasion in close proximity to the connecting tissue almost always results in the infection of the stem tissues of the seedling. Microscopic examinations have frequently revealed bacteria at this point in numbers large enough to produce disintegration of the tissues of the cotyledon and also of the cortex of the stem adjoining this structure, with the formation of large bacterial cavities.

#### VASCULAR INVASION OF THE STEM

As stated above, the bacteria may enter the stem from the cotyledon solely by way of the xylem cells. In such instances the initial invasion is not generally severe and in the early stages the lesion produced at the cotyledonary node is not pronounced, since the organisms do not remain there in large numbers but migrate into the vessels of the hypocotyl and epicotyl. The pathogene is less likely to migrate downward than upward. Microscopic examinations revealed the presence of bacteria in the vessels of the hypocotyl at a distance of 12 to 15 mm from the cotyledons, but below this point they were sparsely observed, and their presence in the xylem vessels of the root has not been demonstrated, although occasionally they have been found in the parenchyma cells.

If proper conditions of moisture and temperature are afforded for the rapid multiplication of the organism, the upward migration may be quite rapid. If the organism penetrates the stem from both cotyledons, many or all of the xylem group of cells may be invaded.

INVASION OF THE PARENCHYMA TISSUE

Penetration of the parenchyma tissue is found in all parts of the seedling *Bacterium phaseoli*, upon entering the cotyledon after germination, may migrate through this structure by way of the intercellular spaces of the parenchyma cells. The bacteria are first seen there in small numbers. Later the mass of bacteria in the intercellular spaces, together with the slime in which they are embedded, may cause the adjacent cells to become greatly distorted (Fig 1, B.)

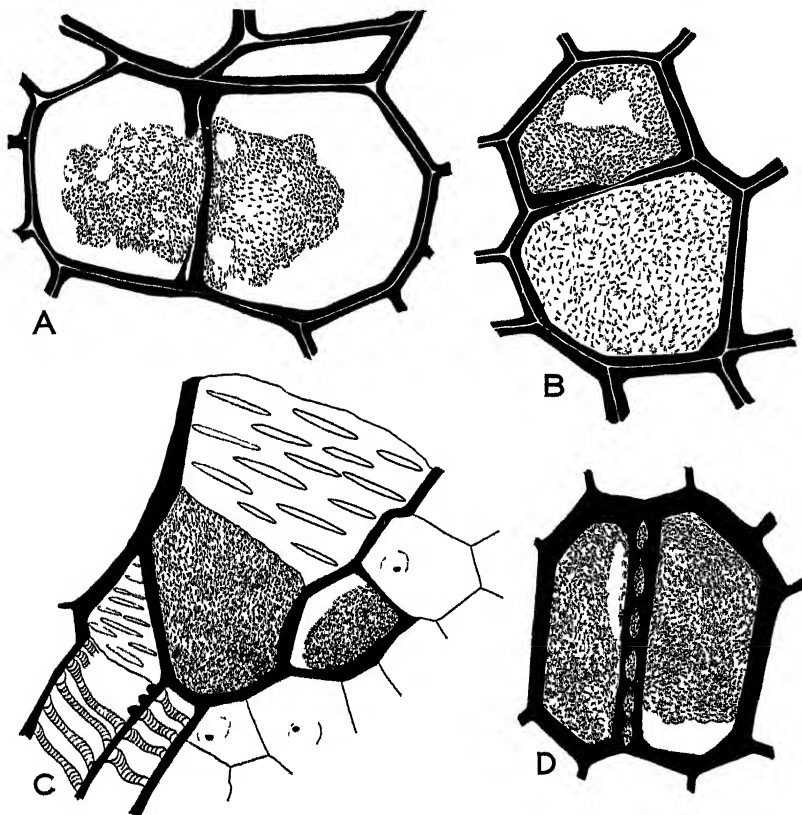


FIGURE 2.—Vascular invasion by *Bacterium phaseoli*.  $\times 800$ . A, The bacteria in the xylem cells are causing a disintegration of the wall of one of the xylem cells and a reduction in the thickness of the adjacent wall, B, an early stage of cell-wall disintegration, the organisms having partially dissolved the dividing walls at the center, C, longitudinal section of a xylem vessel invaded by bacteria, D, xylem cells and pits between the cells invaded by bacteria.

The actual entrance of the bacteria into the cells has not been clearly demonstrated, although intercellular invasion has been observed in numerous instances. The organisms may later involve many cells and finally cause the disintegration of the cell walls, forming lysigenous cavities.

It has been pointed out that the bacteria found in the parenchyma cells of the cotyledon may pass into the tissues of the stem. Here the organisms begin to penetrate the intercellular spaces of the cortical cells (fig. 3, F) in the vicinity of the cotyledonary node. The bac-

If, on the other hand, only one of the cotyledons is infected, a smaller number of xylem cells may be occupied. As the organisms increase in large numbers in the cells, they often break through the cell walls into the adjacent parenchyma tissue, or pass from cell to cell by an apparent dissolution of the cell wall.

In tracing the migration up the stem, bacteria have been observed to fill completely all of the xylem cells of a vascular bundle to such an extent as to give the appearance of a plugging. In the region of the first node where the leaf traces originate, stained sections showed the xylem vessels to be forming a continuous ring, many of which were invaded by the bacteria. (Fig 2, C) In sections closer to the central bud, the xylem vessels were extremely small and were not attacked; however, the vessels of the leaf traces were heavily invaded by the organism.

A drooping of one or both of the primary leaves either at the pulvinus of the petiole or of the leaflet constitutes one of the most striking symptoms found in seedlings infected with *Bacterium phaseoli*. Microscopic examination of sectioned material indicates that because of the succulent nature of the pulvinus the bacteria appear to be more abundant there than in the cells of the stem or petiole. This heavy invasion of the pulvinus may rupture the invaded cells, thus permitting the bacteria to pass into the adjacent parenchyma, with a gradual disintegration of these cells. As a result, the tissues lose their turgidity, causing the leaflet to droop at this region. In the initial stages a drooping of the leaflet takes place during the warmer part of the day, its turgidity being regained at night. After the infection becomes severe the leaflet remains permanently wilted.

Later the bacteria may migrate into the vessels of the petiole. There they frequently rupture the cell walls, pass into the adjacent cortical parenchyma tissues, and invade the intercellular spaces to such an extent that they are extruded from the stomata, producing a bacterial ooze. This condition commonly occurs in diseased seedlings. The organisms eventually enter the vessels of the main veins of the leaf and finally the smaller veins and veinlets. Later they may break out from these cells and produce water-soaked regions extending along the invaded vascular strands. Reddish discolorations of the veins and veinlets are also frequently observed.

The destruction of the growing tip or buds which arise in the axils of the primary leaves is often observed in young infected seedlings. In severely infected plants the death of the buds may occur at the time the seedlings emerge from the soil or after the elongation of the epicotyl. When only the central bud is destroyed, the so-called "snake head" is produced. In such plants new buds often develop in the axils of the cotyledons, and if conditions are unfavorable for the development of the pathogene, the plant, although stunted, may produce a small number of pods. This condition is caused not only by *Bacterium phaseoli*, but, according to Hawley (5), by the seed-corn maggot (*Phorbia fusciceps* Zett.). Harter (4) also found that the threshing operation caused much of this trouble.

As stated previously, the central or axillary buds may be killed after elongation, and in such cases the infection is usually so severe that the plant dies. Bacteria that invade the meristematic tissues of the epicotyl often invade the cells of the growing buds, causing a disintegration of these tissues.

teria often increase in numbers rapidly, spreading the cells apart and later entering them, ultimately resulting in their disintegration and the formation of bacterial cavities

Bacteria frequently break out of severely invaded xylem cells, forming bacterial cavities in the vicinity of the vascular bundles, and from there spread throughout the adjacent tissues by way of the intercellular spaces. In many stained sections bacteria were noted in large numbers, lining the innermost layer of cells surrounding the hollow portion of the pith. It seems reasonable to suppose that the organism could rapidly pass upward throughout the epicotyl in this manner

It has been observed in sections of the epicotyl that the entire cortical tissue was invaded (fig 4), but the xylem cells remained intact. In such instances bacteria were often found to pass out of the stem by way of the stomata (fig 5, B) and produce a bacterial ooze on the surface, which is so often noted on infected plants. Under favorable conditions, the bacteria may enter other stomata and cause infection at other points

#### RELATION OF *BACTERIUM MEDICAGINIS* VAR. *PHASEOLICOLA* TO THE HOST TISSUES

*Bacterium medicaginis* var. *phaseolicola*, after entering the seed by way of either the raphe (fig 6) or the micropyle (fig. 7), invades the cotyledonary cells in much the same manner as *Bact phaseoli*. No entry into this tissue has been found to occur before germination, although bacteria may be found in large masses in the seed coat and also around and between the cotyledons

In slides of stained material, the organisms were found in the xylem vessels of the cotyledon (fig 8 and fig 9, B) which are distributed throughout this structure. In some cases only a few cells of such a vascular bundle were occupied, whereas in other instances many of the cells were filled with bacteria. When abundant, the organism usually caused the disintegration of many of the cells (Fig 9, B)

The organism likewise invades the intercellular spaces of the cotyledonary cells, apparently dissolving the middle lamellae in much the same manner as does *Bacterium phaseoli*. If the infection is severe enough to cause a breakdown of the tissue, large bacterial cavities are produced. Intracellular penetration also occurs

From the cotyledon the bacteria enter the stem of the young seedling either by way of the vascular tissue connecting these two structures or through the intercellular spaces of the parenchyma cells. If the bacteria pass by way of the xylem cells into the epicotyl and hypocotyl, they are then carried to the xylem cells of the stem, causing, in cases of severe infection, a breakdown of the cells (Fig. 9, A.) The organisms do not appear to travel to a great extent in a downward direction, since they are seldom observed in the tissues of the hypocotyl much below the cotyledonary node. The bacteria in the xylem vessels pass upward, increasing in number, and may, if the infection is severe, cause the death of the seedling. They often fail to cause any exterior symptoms for some little time, but later they appear on the young leaf veinlets as water-soaked areas and finally as reddish discolorations. Microscopic examination reveals

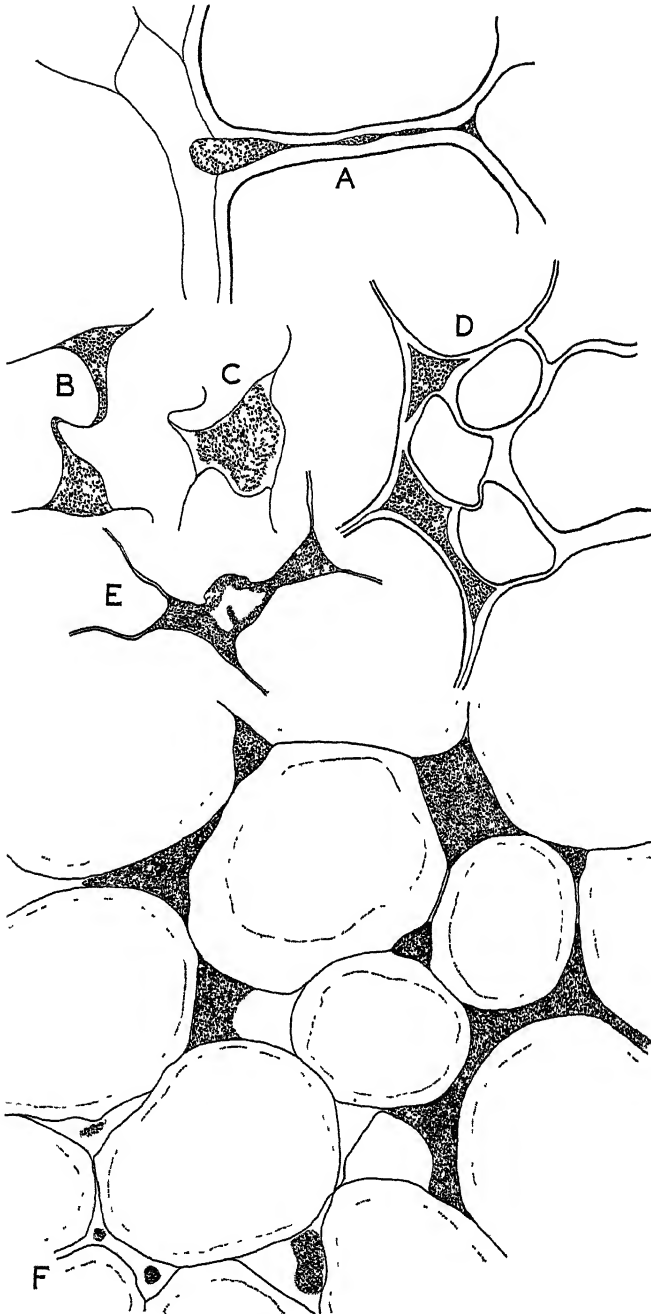


FIGURE 3—Intercellular migration of bacteria in the bean tissues.  $\times 555$ . A, *Bacterium flaccumifaciens* invading the intercellular space between two xylem cells, B, C, D, and E, intercellular invasion of the cortical cells of the stem by *Bact flaccumifaciens*, F, *Bact phaseoli* in the intercellular spaces of the cortex of the stem

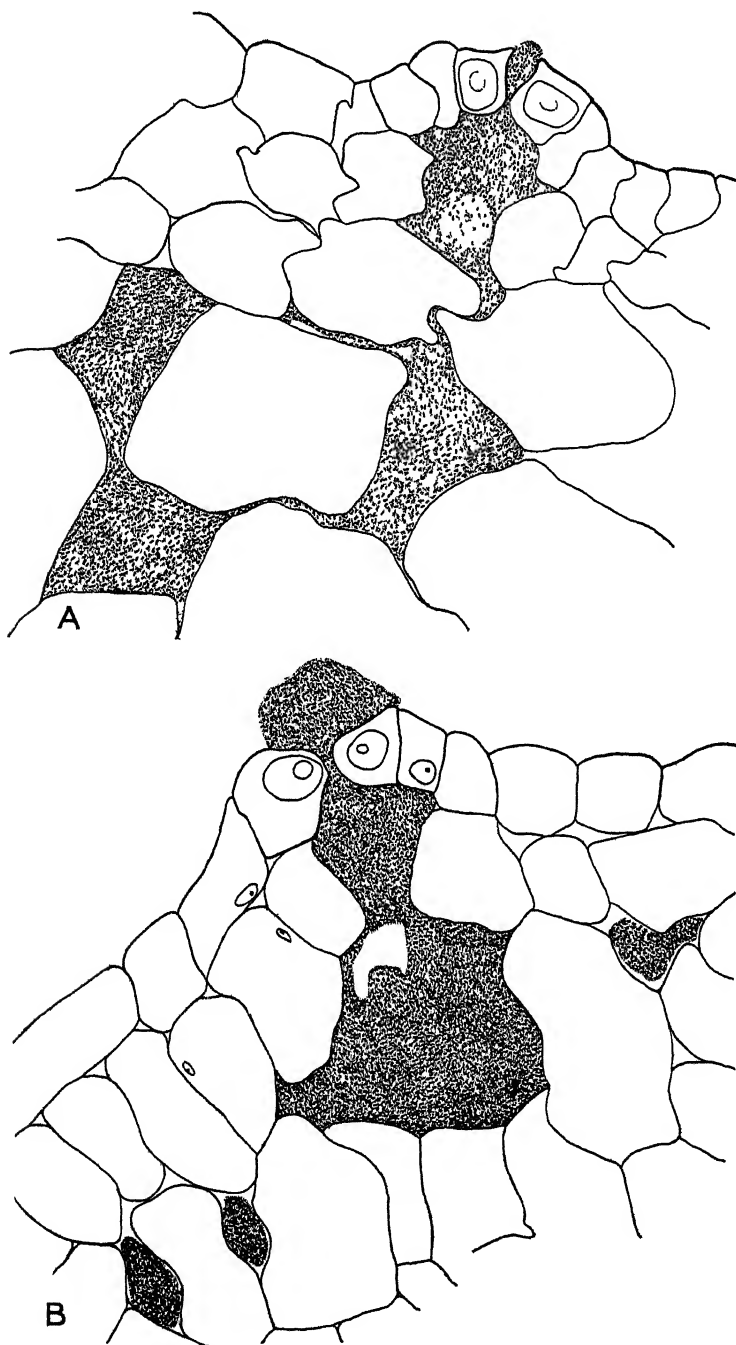


FIGURE 5—Cross section of stem showing bacteria extruding through the stomata  $\times 770$ .  
A, *Bacterium medicaginis* var *phaseolicola*, B, *Bact phaseoli*

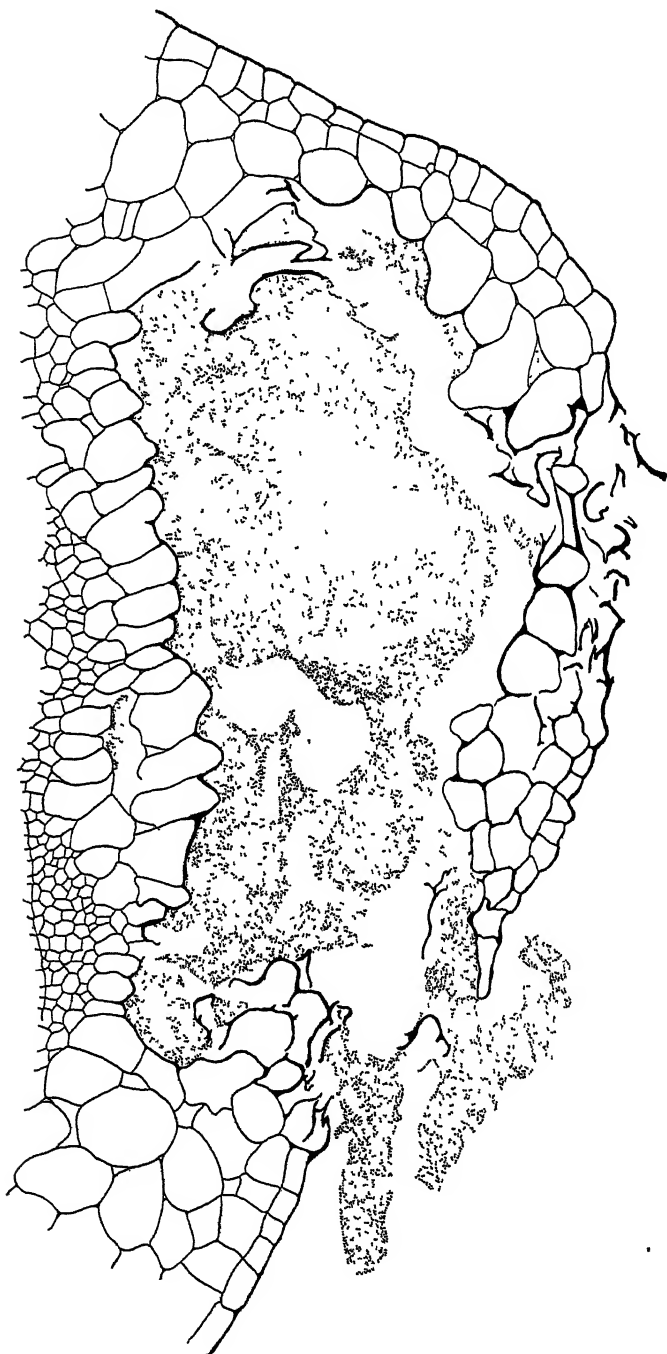


FIGURE 4—Cross section of a portion of the epicotyl showing a lysigenous cavity produced by *Bacterium phaseoli* in the cortex.  $\times 160$

in such cases bacteria in the vessels as well as in the adjacent parenchyma tissue

The vascular tissue of the stems of very young bean plants is not well developed. The xylem tissue consists of small bundles sometimes wholly of either protoxylem or metaxylem cells, although commonly



FIGURE 7—*Bacterium medicaginis* var *phaseolicola* entering the bean seed coats through the micropyle. The large intercellular spaces of this region afford a means for rapid migration of the organism.  $\times 185$

both types are present, the proportions of each varying with the rapidity of growth of the region in question. These cells may vary from two to eight in a bundle and are distributed in a radial manner around the stem. At this stage no secondary thickening has taken place and the cell groups are separated from one another by parenchyma tissue.



FIGURE 6.—Portion of bean seed showing vascular invasion by *Bacterium medicaginis* var *phaseolicola*. The dark-stained masses are bacteria in the xylem cells of the raphe and extend from the funiculus into the integuments.  $\times 150$

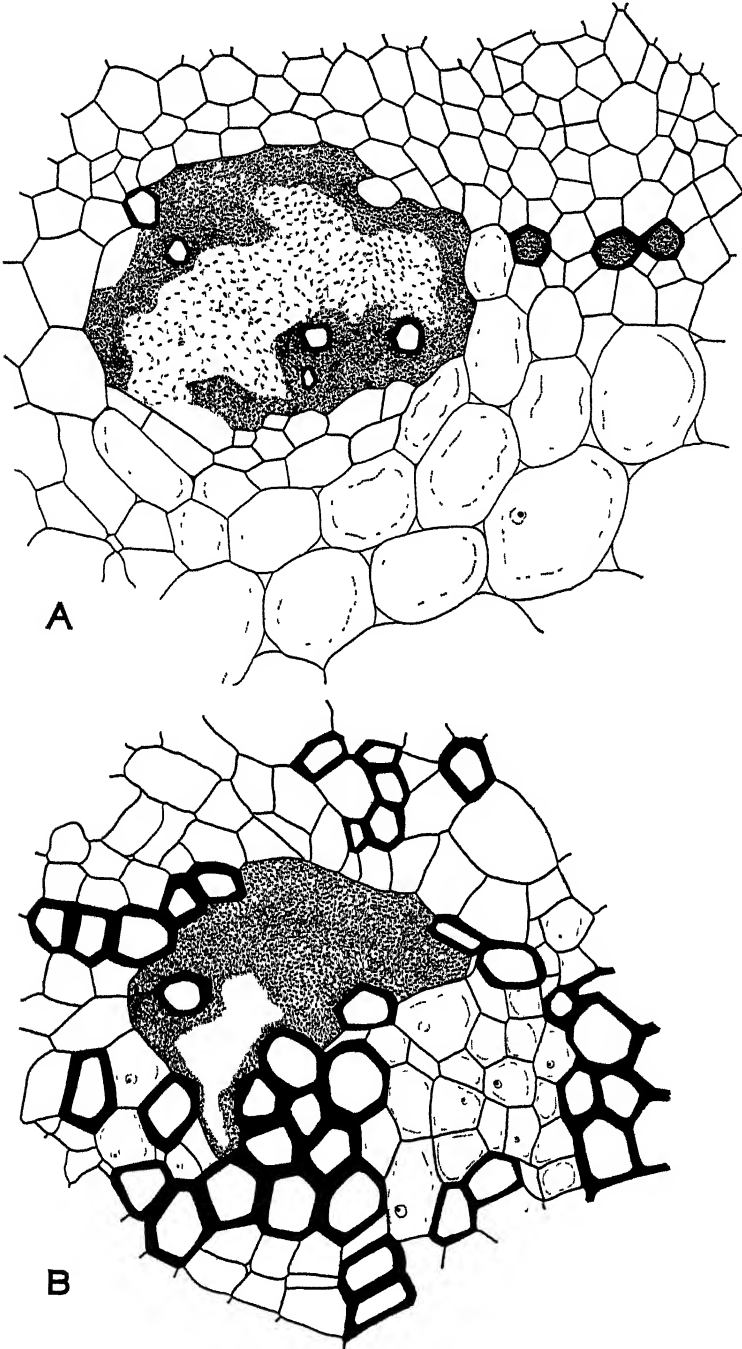


FIGURE 9—Invasion of bean tissues by *Bacterium medicaginis* var *phaseolicola*. A, Cross section of stem near cotyledonary node showing bacterial cavity in the region of a vascular bundle, B, cross section of cotyledon showing bacterial cavity in the region of a vascular bundle  $\times 485$

Stained microscopic sections of the young stem near the region of the cotyledonary node show that *Bacterium medicaginis* var *phaseolicola*, which had invaded many of the xylem cells, had broken out from them and had formed lysigenous cavities in the region of the bundles (Fig 9, A) These cavities were often found around the larger bundles, the smaller ones consisting of a few cells free of bacterial invasion. It seems reasonable to suppose that if more than half of these cells are destroyed, which is often the case, the transpiration stream of the plant is considerably reduced and death of the seedling may result.

The relation of *Bacterium medicaginis* var *phaseolicola* to the parenchyma tissues of the plant is very similar to that of *Bact phaseoli*. The organism is found more frequently in this tissue than in the xylem cells, even though vascular invasion is very common. The pathogene invading the parenchyma tissues by way of the stomata multiplies in the substomatal cavity and then passes throughout

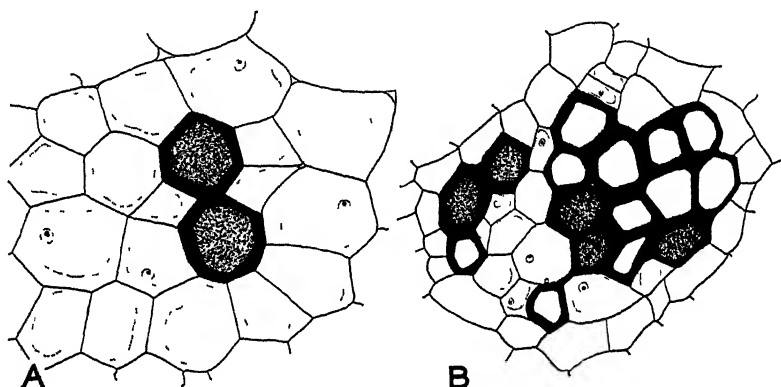


FIGURE 8.—Vascular invasion of the cotyledon by *Bacterium medicaginis* var *phaseolicola*. The bacterial invasion took place after germination of the seed, and the organisms are restricted to the small xylem cells.  $\times 550$

the intercellular spaces of the adjacent cells. The large mass of bacteria, together with the slime in which they are embedded, frequently causes these spaces to become decidedly enlarged with a distortion of the surrounding cells. (Fig 10, B, D, E, and F)

Intercellular and intracellular penetration is commonly observed in the stems of diseased seedlings that are invaded through infected cotyledons (Fig 10, A) Later the bacteria may cause a breakdown of many of the cells, forming bacterial pockets of various sizes. Similar observations have been made in the cortical region of the stem in close proximity to the growing point. In such instances the bacteria may be extruded from the stomata (fig 5, A) or in case of severe infection a rupture of the epidermis may occur, with the production of a bacterial exudate on the surface of the stem.

#### RELATION OF BACTERIUM FLACCUMFACIENS TO THE HOST TISSUES

Like *Bacterium phaseoli* and *Bact medicaginis* var *phaseolicola*, *Bact. flaccumfaciens* enters the ovule through either the raphe or the micropyle. Bacteria entering the vascular connection are carried

mon to find the growing tip destroyed in diseased seedlings, and it is believed that this is caused by the organism in the early stage of seedling development

Seeds infected by the passage of *Bacterium flaccumfaciens*<sup>4</sup> through the raphe seldom produce plants with a diseased epicotyl. The pathogene in such instances enters the seed at the side opposite the embryo and seldom traverses the seed coat to the extent of passing into that part of the seed in close proximity to the young developing seedling. Under conditions of poor germination, where the seed remains in the soil for a number of days, this condition may be brought about, but with normal germination such is probably not the case.

Even though entrance into the embryo has not been observed, nuclear changes appear to take place. In numerous sections of diseased seeds showing the bacteria surrounding the young hypocotyl, the cell nuclei appeared to have been absent. On the other hand, where no bacteria were observed the nuclei were present and apparently normal. It seems reasonable to suppose that the toxic or enzymatic effect of the organism can easily bring about a condition of disintegration and death of the nuclei.

*Bacterium flaccumfaciens* enters the cotyledon at the time of germination in much the same manner as *Bact. phaseoli* and *Bact. medicaginis* var. *phaseolicola*. It then passes throughout the intercellular spaces of the cells and in case of severe infection becomes intracellular. The organisms may then pass into the stem either through the xylem vessels or by way of the parenchyma cells. Severe infection of the cotyledon often causes it to shrivel and fall from the stem. In such cases the region in close proximity to the cotyledonary node is severely invaded by the organisms and under favorable conditions they may cause the death of the plant.

Unlike *Bacterium phaseoli* and *Bact. medicaginis* var. *phaseolicola*, *Bact. flaccumfaciens* does not migrate to any extent in the parenchyma tissues but readily becomes a vascular parasite, invading only the xylem vessels (fig 11, A, C), giving rise to the distinctive wilt symptoms characteristic of the disease. The passage of the organism is primarily in an upward direction; it seldom goes far below the region of entry. The bacteria may not enter all of the xylem bundles of the stem, but may be restricted to a few vessels in the group. If the bacteria multiply rapidly the vessels may become filled and often rupture, thus allowing the bacteria to enter the adjacent parenchyma cells; however, the progress of the organism is slow and seldom spreads to any extent into these tissues. The pathogene, if released from the vascular tissues, may form lysigenous cavities in the region of the invaded vessels (Fig. 12.)

In seedling-infected plants *Bacterium flaccumfaciens* has never been found in the region of the pith and only occasionally in the cortical cells. In plants inoculated artificially with a needle the pathogene was observed in the intercellular spaces of the pith (fig 3, A), where it developed extremely slowly. The organisms were found only in the intercellular spaces at the angles of the cells. These intercellular spaces were not enlarged, nor was the middle lamella dissolved, as was frequently the case in plants attacked by *Bact. phaseoli* and *Bact. medicaginis* var. *phaseolicola*. In the parenchyma the organism is

<sup>4</sup> The culture of *Bacterium flaccumfaciens* used in these investigations was furnished by Florence Hedges

into the seed coats, where often, because of the large intercellular spaces, migration may be rapid. Entrance through the micropyle enables the bacteria to traverse the region in close proximity to the growing tip of the hypocotyl or the epicotyl. Penetration of the

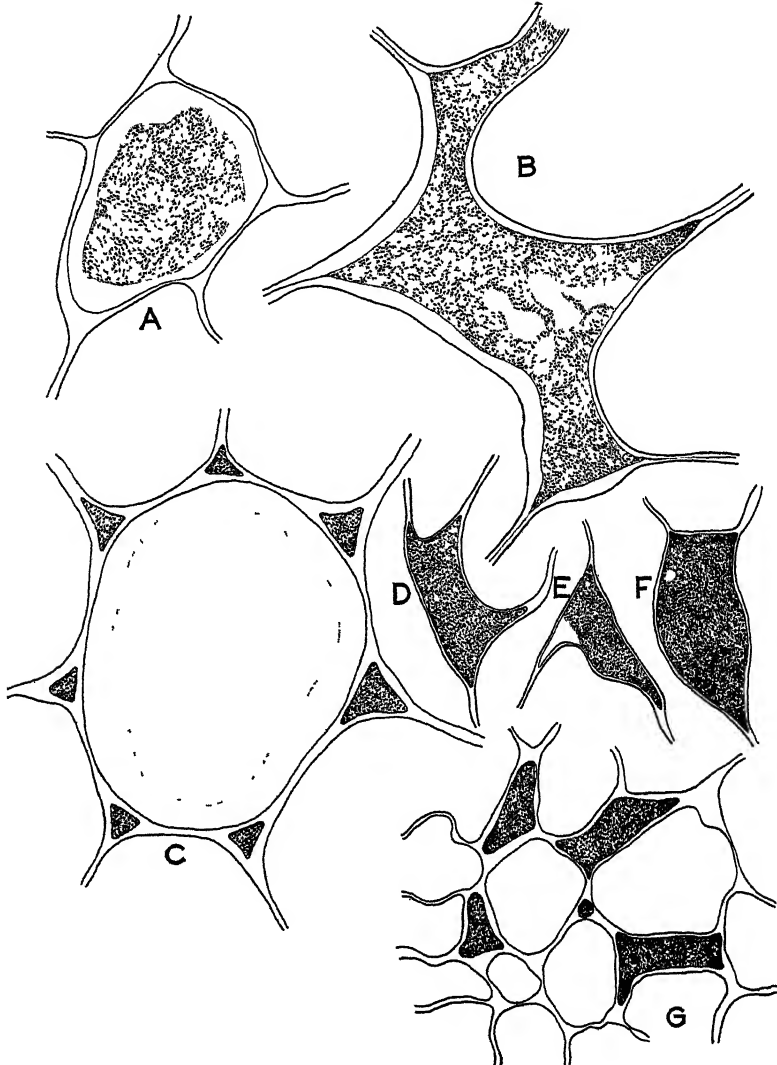


FIGURE 10.—Intracellular and intercellular invasion of the parenchyma tissues by *Bacterium medicaginis* var *phaseolicola* in the region of the cotyledonary node.  $\times 750$ . A, Intracellular penetration of a pith cell. B, D, E, and F, Intercellular penetration of pith cells. In some cases the bacteria together with the slime in which they are embedded have produced an enlargement of the intercellular space, C and G, Intercellular penetration of cortical cells.

pathogene into either the hypocotyl or the epicotyl has never been observed, but it is presumed that as growth takes place the bacteria may enter them or affect them through toxins or enzymes in such a way as to cause imperfect development or death. It is not uncom-

ordinarily found in the vicinity of invaded xylem cells, where it has either dissolved or broken the cell wall (Fig 12) In its upward migration *Bact flaccumfaciens* may invade the young growing bud and cause its death. It may likewise enter one or both of the petioles through the leaf pulvinus, filling the xylem cells (fig 13), and in case

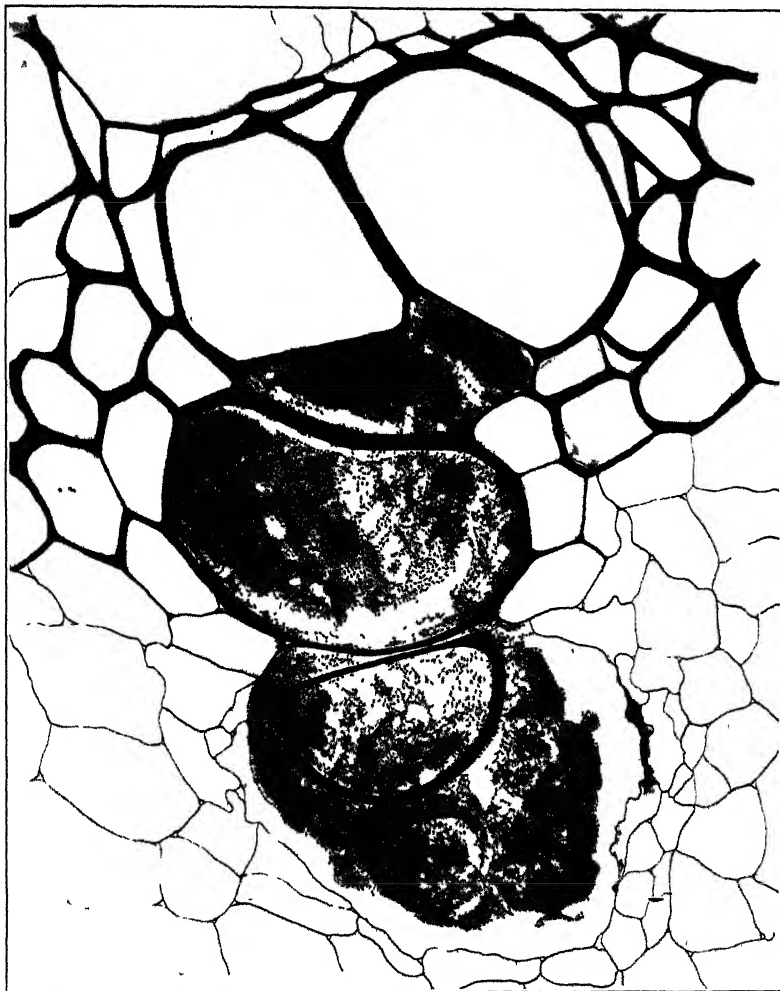


FIGURE 12—Cross section of stem showing *Bacterium flaccumfaciens* in the xylem vessels. As a result of cell-wall dissolution, the bacteria in the lowermost cell have been released, producing a lysigenous cavity. The adjoining cell above has become separated from the other vascular cells by a dissolution of the middle lamella. Retouched photomicrograph  $\times 800$

of severe infection, cause a drooping of the leaf. This condition may continue for a number of days, after which the leaflet remains flaccid and finally dies. Many stained sections have shown severe invasion of the pulvinus, and in numerous instances bacteria have caused a breakdown of the xylem cells. Bacterial migration then progresses

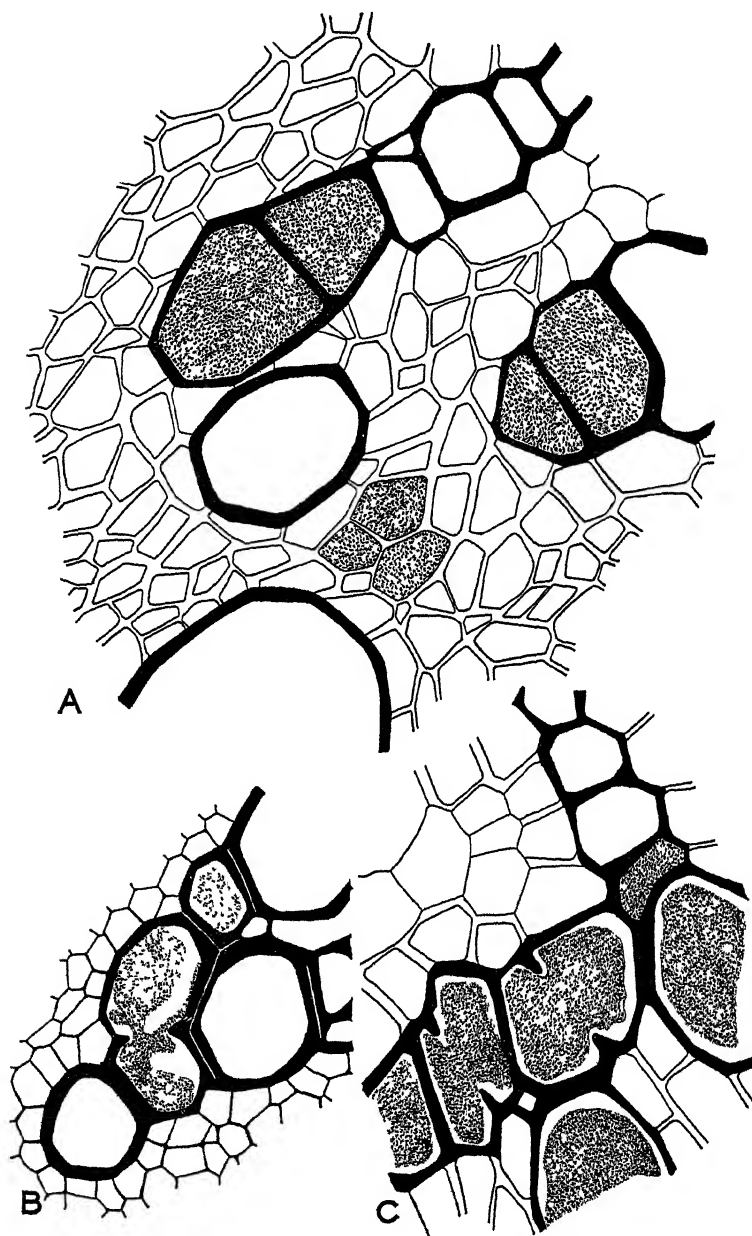


FIGURE 11—Vascular invasion of bean tissues by *Bacterium flaccumfaciens*  $\times 585$  A and C, Cross section of a stem showing bacteria in both primary and secondary xylem vessels. In C the cross walls have been disintegrated through bacterial action. B, Cross section of pulvinus of petiole showing disintegration of the cross walls separating two invaded cells.

them even though they were open. Entry was gained only through an injury to the stem or leaf. If the inoculating needle only penetrated the cells of the cortex, the bacteria would make little progress and generally the plant would outgrow this infection if proper conditions were afforded for good growth. If, on the other hand, the organism was injected into the vessels of the young stem, the bacteria would multiply rapidly and under proper conditions would cause a wilting of the young plant.

Most rapid progress of the organism was made when young pods were inoculated along the dorsal suture. Seldom would the organism produce any decided external symptom, but on opening the pod it would be found that most of the seeds were severely invaded by the organism, in some cases to such an extent that they failed to mature.

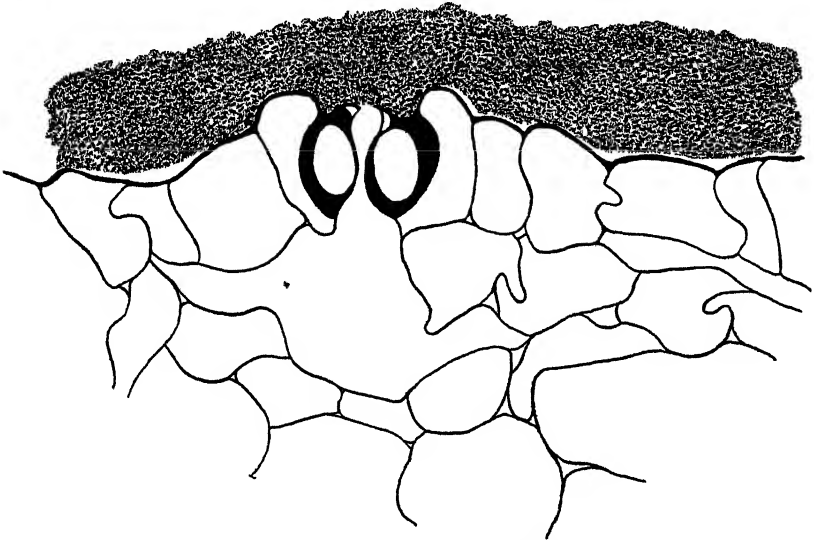


FIGURE 14.—Cross section of a stem showing *Bacterium flaccumfaciens* massed on the exterior over an open stomata. Penetration through the stomata by this organism has never been observed.  $\times 800$

#### CELL-WALL DISINTEGRATION THROUGH BACTERIAL ACTION

Cell-wall disintegration by phytopathogenic bacteria has been given little investigation in the past, although considerable evidence has been published on the dissolution of the middle lamella. As early as 1879 Van Tieghem (14) studied the decomposition of various vegetable tissues, considering this action as due to a species of *Bacillus* to which he gave the name *Bacillus amylobacter*. He found that only the young tissues were decomposed, while the older ones, which were lignified, cuticularized, or suberized, were resistant to the action.

Kramer (9) isolated from decaying potatoes an aerobic sporiferous bacillus which was capable of dissolving the intercellular substances and attacking the cellulose membrane.

Bacteria of a saprophytic nature associated with the rotting of potatoes were studied by Wehmer (15), who found two types of decomposition, one in which the middle lamella only was dissolved and the other in which there was an ultimate dissolution of the entire wall. He presumes that an acid rather than an enzyme produced by the bacteria may be the agent in the solution of pectic compounds.

into the xylem cells of either one or both petioles, thence into the leaf veins and veinlets

*Bacterium flaccumfaciens* may cause small water-soaked spots on the young primary leaves, but these lesions are not produced by the entry of the organism into the stomata but by being exuded from them. One important difference between *Bact flaccumfaciens* on the one hand and *Bact phaseoli* and *Bact medicaginis* var *phaseolicola* on the other is that the former does not invade the stomata, whereas

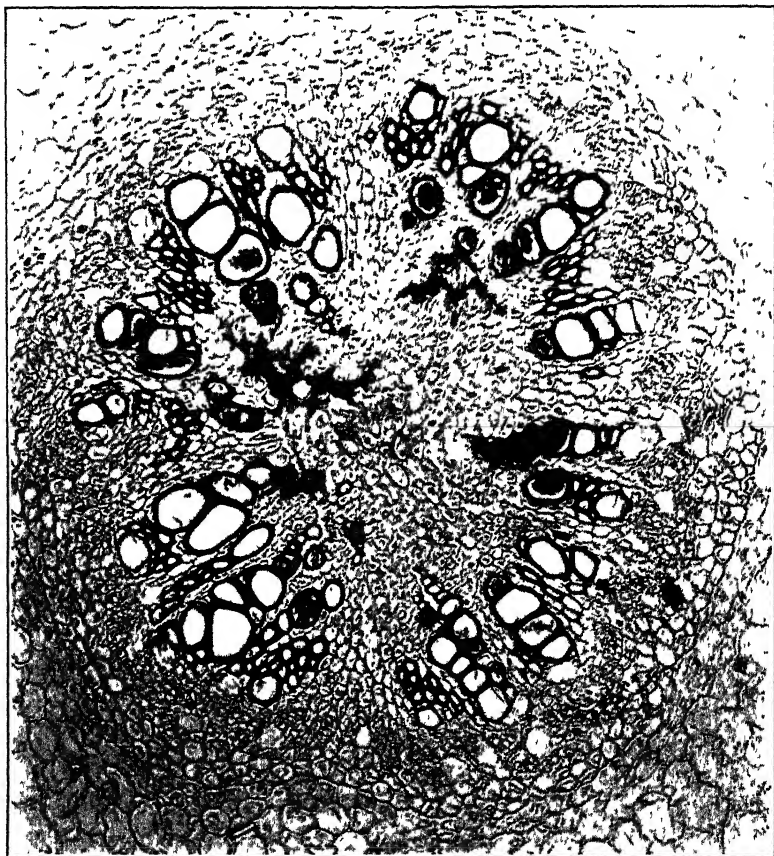


FIGURE 13.—*Bacterium flaccumfaciens* invading the cells of the pulvinus of the bean petiole. The dark-stained masses of bacteria are seen in a number of the xylem cells. Most of the invasion in this region is vascular.  $\times 165$

the latter two do and pass rapidly through the intercellular spaces of the cells surrounding the substomatal cavities. *Bact. flaccumfaciens* when sprayed on plants and placed in a moist chamber of saturated humidity for 24 hours did not enter the stomata of the inoculated leaves or stems (fig. 14), while *Bact. phaseoli* and *Bact. medicaginis* var *phaseolicola* entered these openings under similar conditions. Histological sections of material inoculated with *Bact. flaccumfaciens* showed the bacteria massed over the stomata, but they never entered

When the organisms are found in large numbers invading any particular tissue, they are always embedded in a slimy matrix with high absorptive qualities. With the absorption of liquids from the surrounding cells, a pressure may be produced sufficient to cause the cells to become obviously ruptured. Destruction of wall material by this means often occurs, but it does not appear to be the only explanation, since many sections of stained material have shown a partial to an almost complete dissolution of the walls, accompanied by only a moderate number of bacteria. Most likely a combination of both of these factors often occurs.

#### WALL DISINTEGRATION IN PARENCHYMATOUS TISSUE

The dissolution or disappearance of wall material in cells of the parenchyma, either of the pith or of the cortex, was noted with all three organisms, but more particularly with *Bacterium phaseoli* and *Bact. medicaginis* var *phaseolicola* than with *Bact. flaccumfaciens*. Large lysigenous cavities were often seen in the region of the cotyledonary node (fig. 9, A), where the bacteria invaded the stem from the cotyledons, and also in the epicotyl (fig. 4) in the region of the growing tip. Here large bacterial cavities were observed with the cell walls completely broken down. The disappearance of the walls could not be entirely accounted for by a crushing of the cells by mass action of the bacteria embedded in a slime, since, if this were the only cause, the remnants of the broken cell walls could have been seen throughout the bacterial cavity. A solvent action by a substance produced by the bacteria was probably more responsible for the formation of these cavities. Only occasionally could cell-wall fragments be found. Often, if the invasion was in the cortical tissues, the bacteria would break out from the epidermis (fig. 4), forming a bacterial ooze. It is believed, however, that this exudation was due to internal pressure rather than to dissolution, since the outer walls of the epidermis are composed of suberin and cutin, substances that are possibly resistant to bacterial action.

The parenchyma which surrounds invaded xylem cells likewise disappears if the bacteria in the xylem are released. (Fig. 12) Innumerable instances of this have been observed with the three organisms. *Bacterium flaccumfaciens* seems to produce a smaller bacterial cavity than the other two and is limited to the region surrounding the invaded xylem. (Fig. 12)

As previously reported (17), *Bacterium phaseoli* when in the parenchyma tissues first invades the intercellular spaces and, with the possible production of a pectin-dissolving enzyme, attacks the pectic compounds that cement the cells together. With the increase in the number of bacteria the intercellular spaces are greatly enlarged, often causing a distortion of the adjacent cells (Fig. 1) It is supposed that the hemicelluloses that make up the layer of the cell wall adjacent to the pectin materials may later be dissolved through enzymatic activity, after which the cellulose is finally attacked. It appears that after the organism penetrates the cell wall, dissolution of its component materials goes on at a more rapid rate.

#### WALL DISINTEGRATION IN VASCULAR TISSUE

A more detailed study of cell-wall destruction was made on xylem tissue because various gradations from a partial (fig. 2, A, B) to an

Jones (8), working with *Bacillus carotovorus*, the cause of the slimy soft rot of vegetables, showed that the organism produces an enzyme, pectinase, which causes the dissolution of the middle lamella of the parenchyma cells, but in no case did he find evidence of such action upon lignified or cuticularized walls.

Smith (13) states, regarding *Pseudomonas campestris* E. F. Smith, that the cell walls of invaded cells become vague in outline and finally disappear. Drechsler (3), working with the same organism, observed the formation of bacterial cavities, and illustrates the apparent disintegration of cell walls of both parenchyma and xylem cells. Skorice (12), working with *Bacterium pisi* Sack, likewise found evidences of wall disintegration and possible dissolution. He suggests that the bacteria, together with the slime in which they are embedded, rupture many of the invaded cells, although some of the large cavities found in the parenchyma of the cortex and pith can not be entirely explained by rupturing and crushing of the tissue. He believes that parts of the cavity at least appear to result from solvent action of the organism.

Nixon (11) in his studies on the migration of *Bacillus amylovorus* Burr. remarks that the organism has the power to dissolve the cell wall. His evidence with stained microscopic preparations proved that openings in the cell wall were formed by a dissolution of the wall by the organism.

In histological studies of fire blight of apple, Miller (10) observed *Bacillus amylovorus* migrating from one cell to another through openings which appear to have been formed by a dissolution of the wall substances. He ascribes this phenomenon in some cases to an internal pressure due to mass action and in others to a dissolving action on the cell walls. He suggests the possibility of an enzyme of the nature of cellulase being secreted in small amounts.

In a previous paper (17) the writer reported the disappearance of all or a part of the cell wall when invaded by *Bacterium phaseoli*. It was believed that the cellulose walls disappear, leaving only the lignified structures. Since then these studies have been continued not only with *Bact. phaseoli*, but also with *Bact. medicaginis* var. *phaseolicola* and *Bact. flaccumfaciens*. Throughout the course of the study more instances of the disappearance of cell-wall material have been noted with *Bact. phaseoli* and *Bact. medicaginis* var. *phaseolicola* than with *Bact. flaccumfaciens*. Particularly is this true in the case of the parenchyma tissues, where *Bact. flaccumfaciens* was found only in close proximity to broken invaded xylem vessels. In the following discussion the organisms will not be considered separately, since dissolution was noted with all three parasites.

Many different stages of cell-wall disintegration were observed throughout the study. The fact that only bean seedlings were used in the investigation may account for the observance of much of this wall disappearance, since little secondary wall formation was in evidence at the time the preparations were made. In this condition the cells consist mostly of celluloses and hemicelluloses, which are more readily dissolved through chemical action than is lignin, which makes up much of the secondary wall structure. The disappearance of the cell walls was noted not alone in parenchyma tissues of the pith and cortex, but also in the xylem vessels, where infection was severe.

migration might be attributed to this, it is believed that disappearance of the cell wall resulted either through physical mass action of the bacteria or by a dissolution of the walls. If only advanced stages in the disappearance of the walls were noted (fig 11, B, C, fig. 15) in which only the "peglike" edges of the walls of the invaded cells remained, the supposition of natural passage of the organism from cell to cell through normally broken connecting walls might have explained all bacterial migration. Since, however, various stages in the disappearance of the wall were seen (fig 2, A, B, fig 16), it must be assumed that more than one explanation is necessary to account for the passage of the organism from one cell to another (fig 15) within the vascular bundle or into the surrounding parenchyma tissue. (Fig 12)

That wall dissolution by bacteria proceeds at different rates in different invaded cells is evident from the fact that where two contiguous cells are affected the rate of action on one dividing wall may be faster than that on the other (Fig 2, B). Stained sections of material infected with *Bacterium phaseoli*, in which two adjacent cells were equally invaded, have shown this to be the case in both initial and advanced stages of wall destruction. In an early stage a slight corrosion of one of the dividing walls was seen, while the opposite wall appeared to be less dissolved. Similarly, in a more advanced stage one wall appeared to be almost entirely dissolved while that of the adjacent cell remained intact (Fig 2, A)

Microscopic evidence indicates that dissolution begins on the inner dividing walls of invaded xylem cells and rarely if ever on the outer walls (Fig 16). The lamellae of the outer cell walls, as well as the walls themselves, even though the cells are invaded by bacteria, stain deeply; whereas the dividing walls of these cells stain weakly and the middle lamellae are barely visible. The materials composing the cell walls may be altered by the bacteria in such a way as to change their staining reaction. Weakly stained walls appear to indicate the beginning of dissolution, while those deeply stained furnish evidence of no apparent action.

Later the walls separating the two cells are reduced in thickness, especially at the center, while at the outer corners they appear normal. Very early stages of such a condition have been observed where the only noticeable evidence was a slight dissolution of the wall. (Fig 2, B) Finally the organism or the dissolving principle may produce a small opening in either or both of the walls that divide the invaded cells (Fig 16). The organism may then dissolve the pectic materials composing the middle lamella. Lamellation of these walls occurs next, giving them the appearance of being shredded (Fig. 16). With the passage of the organism from cell to cell and further dissolution, the ends of the disintegrated wall become rounded (fig 11, B, C, fig. 15), which suggests that the process is one of dissolution and not a cell rupture due to an internal pressure set up by the bacteria and the slime in which they are embedded. If the latter were solely the cause, the ends of the wall would be ragged and not smooth as they usually are.

In instances of severe invasion of most or all of the cells of a vascular bundle the walls of entire cells appeared to have been dissolved. (Fig. 9, A, B.) Many similar observations were made in microscopic sections of the epicotyl. Apparently not all of this dissolution began from the inside of the cells, but after the organism had

almost complete disappearance of the cell wall was noted. (Fig. 11, B, C; fig 15) Jones (?) found in working with *Aplanobacter insidiosum* McC that there exists an open communication between vessels in the alfalfa root through which bacteria may pass without actually

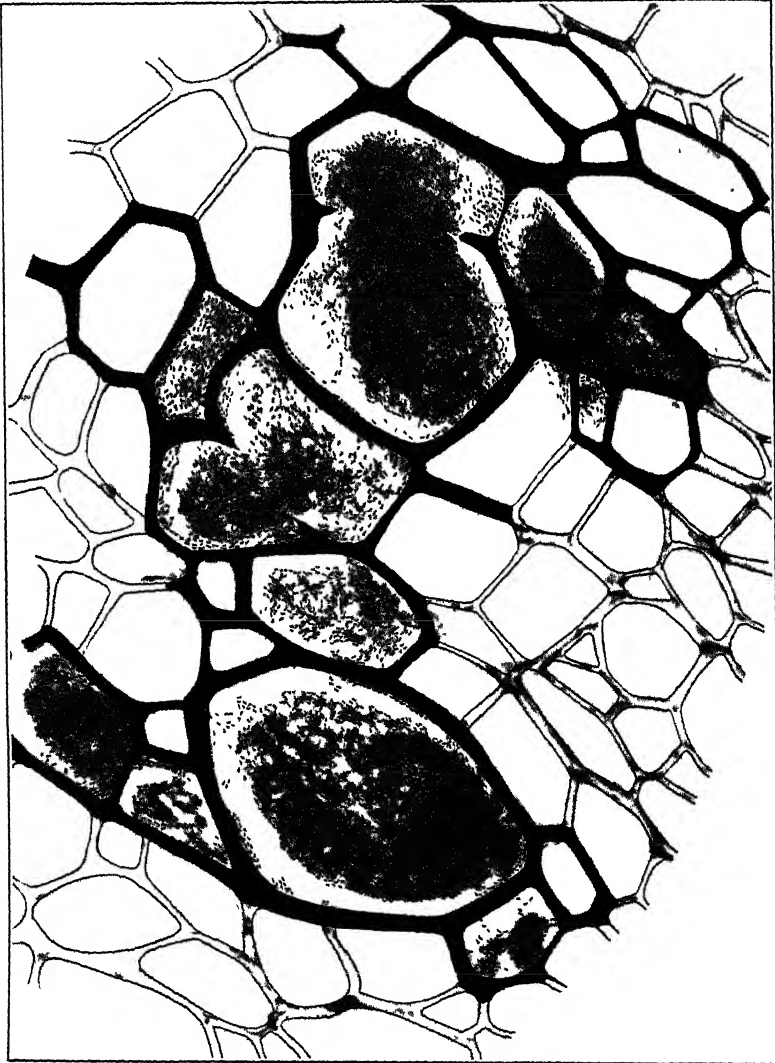


FIGURE 15—Vascular invasion by *Bacterium flaccumfaciens*. The bacteria are seen in most of the xylem cells of the vascular bundle and in two instances are passing from cell to cell through broken cell walls. Retouched photomicrograph  $\times 960$

penetrating cell walls. He states that the bacteria are distributed around the circumference of the roots to some degree if not entirely through these open vascular connections.

Such openings have been seen in stained sections of the bean stem, although they were not commonly found. While some bacterial

The bacteria that collect in large masses around xylem cells may, by a dissolution of the middle lamellae, cause these cells to become separated from one another. Such a case is shown in Figure 12. Here the bacteria have entirely dissolved the walls of the lowest cell of the vascular bundle, resulting in the formation of a cavity of considerable size in the parenchyma tissue. As a result of the dissolution of the middle lamella the next cell above finally became separated from its adjacent cell. Many cases have been noted where cells were unattached in lysigenous cavities. The walls of such cells usually stain weakly in comparison with those that are normal or only slightly invaded. Usually they are thinner, which suggests that they are partially dissolved.

#### SUMMARY

From the standpoint of symptomatology it appears possible to differentiate the three diseases caused by *Bacterium phaseoli*, *Bact medicaginis* var *phaseolicola*, and *Bact flaccumfaciens*. Histologically, the first two diseases can be readily distinguished from *Bact flaccumfaciens*, but since *Bact phaseoli* attacks the host tissues in a similar manner to *Bact medicaginis* var *phaseolicola*, it is often difficult to determine the exact organism except through cultural methods.

Because of the fact that it is Gram-positive, *Bacterium flaccumfaciens* can readily be differentiated in the host tissues from *Bact. phaseoli* and *Bact medicaginis* var *phaseolicola*, both of which are Gram-negative. Furthermore, *Bact. flaccumfaciens* is primarily a vascular parasite invading only the xylem vessels, whereas *Bact. phaseoli* and *Bact medicaginis* var *phaseolicola*, even though they are found in the xylem cells very frequently, appear to show a preference for the parenchyma tissues. Migration of *Bact flaccumfaciens* into the intercellular spaces of the parenchyma is very slow. It is usually limited to those cells in close proximity to invaded xylem cells that may have been ruptured by an internal bacterial pressure or by cell-wall dissolution.

Another important difference between *Bacterium flaccumfaciens* and the other two parasites is that the former does not invade the stomata (fig. 14), whereas the latter do, and pass rapidly through the intercellular spaces of the cells surrounding the substomatal cavities. *Bact flaccumfaciens* makes the most rapid progress when the pods are inoculated along the dorsal suture.

The migration of *Bacterium phaseoli* and *Bact medicaginis* var. *phaseolicola* in the plant tissues is very similar. Both organisms penetrate the seed and enter the stem in the same manner. They attack the parenchyma and vascular tissues similarly, possibly having a slight preference for the parenchyma cells. They migrate through the intercellular spaces of the parenchyma, dissolving the middle lamella slightly in advance, with a subsequent collapse of the invaded cells. The xylem cells are attacked by both organisms, but the bacteria are not restricted to them. These organisms when in large numbers may rupture or dissolve the cell walls and pass into the adjacent parenchyma tissues of the pith or cortex, where they multiply rapidly when conditions are suitable for their development. Both organisms may pass throughout the cortical tissues of the stem, emerge from the stomata, and enter other stomata when conditions are suitable for their dissemination.

been liberated from the invaded cells, either by dissolution or by a rupture of the walls, a lysigenous cavity was produced around the vascular bundles, resulting in a breakdown of the surrounding parenchyma tissue (Fig 12)



FIGURE 16—Disintegration of the bean cell wall by action of *Bacterium flaccumfaciens*. The organism is noted passing from cell to cell, the cross walls of which appear to have been attacked by it  $\times 1,500$

The protoxylem cells, which apparently are composed of more soluble material than the cells of the meta or secondary xylem, appear to show the greatest amount of dissolution. (Fig. 11, B, C; fig. 15) Their walls are often almost indistinguishable, and in some cases entirely so.

# A STUDY OF SAMPLING TECHNIC WITH SUGAR BEETS <sup>1</sup>

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## INTRODUCTION

In plot experiments with sugar beets (*Beta vulgaris* L.) it is customary to weigh all the beets from the plot in order to determine the yield. Sometimes the border rows are removed to eliminate border effect, in other instances border effect is ignored. Except in very small plots, it is not practical to analyze all the beets in order to determine the percentage of sugar. It is imperative, therefore, to resort to sampling methods in selecting the beets for sugar determinations. The percentage of sugar obtained from the sample is considered an estimate of the average sugar percentage of all the beets in the plot.

Johnson <sup>3</sup> studied the variation in sugar percentage from samples of beets taken from different plots. He concluded that 10 beets do not give a valid estimate of the actual sugar percentage in a plot and that a sample of 50 beets would not be too large.

Pack <sup>4</sup> studied the correlation of weight with sugar percentage of individual sugar beets and found the correlation to be negative. He also reviewed other work on this subject and noted that negative correlations were found in the majority of cases.

Clapham <sup>5</sup> and Wishart and Clapham, <sup>6</sup> using the "analysis of variance" method in studying sampling technic with small grains and potatoes, respectively, concluded that this method could be used satisfactorily on the larger plots if the samples were taken in such a manner that the data would lend themselves to adequate statistical analysis.

The writer has applied the analysis of variance method to studies of sampling technic in relation to the estimation of sugar percentage in sugar beets, and the results are presented herein.

## MATERIALS AND METHODS

A small field of sugar beets of the Pioneer variety at the southeast experiment station, Waseca, Minn., was chosen for the experiment. The field had been cropped in a uniform manner in previous years,

<sup>1</sup> Received for publication Nov. 5, 1931, issued May, 1932. Contribution from the National Research Council and from the U. S. Department of Agriculture in cooperation with the Minnesota Agricultural Experiment Station.

<sup>2</sup> Fellow of the National Research Council. The writer takes great pleasure in expressing his indebtedness to Dr. R. A. Fisher, chief statistician of the Rothamsted Experimental Station, Harpenden, Herts, England, under whose guidance this study was made, and to Dr. J. Wishart for help given during the course of the study.

<sup>3</sup> JOHNSON, S. T. A NOTE ON THE SAMPLING OF SUGAR BEET. *Jour. Agr. Sci. [England]* 19: 311-314. 1929.

<sup>4</sup> PACK, D. A. SELECTION CHARACTERS AS CORRELATED WITH PERCENTAGE OF SUCROSE, WEIGHT, AND SUCROSE CONTENT OF SUGAR BEETS. *Jour. Agr. Research* 40: 523-546, illus. 1930.

<sup>5</sup> CLAPHAM, A. R. THE ESTIMATE OF YIELD IN CEREAL CROPS BY SAMPLING METHODS. *Jour. Agr. Sci. [England]* 19: [214]-235. 1929.

<sup>6</sup> WISHART, J., and CLAPHAM, A. R. A STUDY IN SAMPLING TECHNIQUE: THE EFFECT OF ARTIFICIAL FERTILISERS ON THE YIELD OF POTATOS. *Jour. Agr. Sci. [England]* 19: [600]-618. 1929.

The three organisms show very little variation in their ability to dissolve the cell walls. When in mass they are embedded in a slimy matrix which has a high absorptive power that enables it to rupture the cell walls mechanically. The ability to cause disappearance of the cell wall, presumably through dissolution by some enzyme, is common to the three species.

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## EXPERIMENTAL RESULTS

## REGRESSION OF SUGAR PERCENTAGE ON WEIGHT

It is of interest (1) to investigate the regression of sugar percentage on weight, (2) to find whether or not this regression is linear, and (3) if it is not linear, to determine the exact relationship. This may be done from an analysis of variance of sugar percentage and of weight and an analysis of covariance of weight and sugar percentage. Table 1 gives the analysis of variance of sugar percentage.

TABLE 1—*Analysis of variance of sugar percentage*

Variation	Degrees of freedom	Sum of squares	Mean square <sup>a</sup>	Standard deviation	<i>z</i> <sup>b</sup>
Between plots.....	99	384.3977	3.8828	1.9705	} 0.2985
Within plots.....	900	1,923.6990	2.1374	1.4620	
Total.....	999	2,308.0967	2.3104	1.5200	-----

<sup>a</sup> Mean square or variance ( $S D^2$ ) =  $\frac{\text{Sum of squares}}{\text{Degrees of freedom}}$

<sup>b</sup> *z* = one-half the difference between the natural logarithms of the two variances, or the difference between the natural logarithms of the standard errors (standard deviations)

Since there were 1,000 beets analyzed, the total number of degrees of freedom is 1,000 - 1, or 999. The 100 plots contribute 99 degrees of freedom. The remainder, 999 - 99, or 900, is the number of degrees of freedom due to variation within plots. This value might be determined in another way. Since there are 10 beets in each plot, there would be 9 degrees of freedom within each plot. One hundred such plots would give the 900 degrees of freedom allotted to variation within plots.

The total sum of squares is found by squaring the sugar percentages of each of the 1,000 beets and subtracting the product of the general sum multiplied by the general mean. The sum of squares due to variation between plots may be found most easily, when a calculating machine is available, by squaring the sums of the sugar percentages for each plot, dividing by the number of elements contributing to each sum (10 in this case), and subtracting the product of the general sum multiplied by the general mean used above. The sum of squares due to variation within plots is obtained by subtracting the sum of squares due to variation between plots from that of the total. The analysis is then on a single-beet basis. All analyses of variance given in this paper will be on that basis unless otherwise stated.

The significance of the difference between the variance between plots and the variance within plots is found by Fisher's *z* test. *z* is one-half the difference between the natural logarithms of the two variances (or the difference between the natural logarithms of the standard errors). Fisher <sup>8</sup> has provided tables showing the values of *z* that would be attained by chance for two different levels of significance, the 5 per cent and the 1 per cent points. If the 5 per cent point of *z* is exceeded, it is understood that a difference as great as that between the two observed variances would occur by chance between two samples from homogeneous material less than once in 20

<sup>8</sup> FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed 3, rev and enl, 283 p., illus, Edinburgh and London 1930

and cultural conditions during the growing season were similar throughout the field. The beets were grown in rows 22 inches apart and spaced 12 inches apart within the row. The study, therefore, is based on a uniformity trial.

At harvest time the field was divided into plots 33 feet long, and alleyways 2 feet wide were cut at right angles to the rows in order to separate the plots and to prevent beets from one plot from being mixed with those from neighboring plots. All beets adjacent to noticeable skips were removed before harvest, leaving only those plants to be harvested that had normal competition on each side. Ten such rows were chosen for sampling, each 10 plots in length (33 feet), separated from one another by 3 intervening rows. The area might therefore be considered a 10-by-10-plot field, each plot consisting of 4 rows, only 1 of which was sampled. Ten beets were selected from each plot in such a way that the entire length of the row was sampled uniformly. The beets from each plot were lifted by hand, numbered, and put into a properly labeled waterproof bag. The bags were taken to the laboratory and the beets scrubbed clean of dirt. Each beet was then weighed and the percentage of sugar obtained. In analyzing for sugar each beet was bored at an angle through the center. The sugar percentage in the pulp was determined by the cold-water digestion method. The harvesting period was completed in two days and the sugar analyses in three.

The statistical technic employed in this study is known as the analysis of variance. This method was devised by Fisher and first published in complete form in 1923.<sup>7</sup> It has been applied to numerous statistical problems and has proved to be the most flexible and efficient method yet devised for agronomic problems that are to be subjected to statistical inquiry. The method will be explained in some detail in connection with the analysis of results given herein.

The principle of the analysis of variance method is that the total variation between the individual results in a set of data can be separated into a number of parts. If the variation is measured in terms of sums of squares of the deviations of observed values from their mean, it is possible to apportion fractions of the total sum of squares to various known causes, leaving a residual fraction attributable to uncontrolled factors. The mean value of the sum of squares ascribed to any factor (the variance, or standard deviation squared) is obtained by dividing the sum of squares by the appropriate number of degrees of freedom, where the term "degrees of freedom" is used in the sense of "independent comparisons." Thus, with  $n'$  quantities, whose mean is fixed, there will be in general  $n' - 1$ , or  $n$ , degrees of freedom.

Since tests of significance used in the analysis of variance are made on the variance (standard deviation squared) or on the standard error (standard deviation), the standard error instead of the probable error will be used in interpreting the results. The standard error is the common measure of variation used by European statisticians, and with the increased use of the analysis of variance it will probably become more common in North America.

<sup>7</sup> FISHER, R. A., and MACKENZIE, W. A. STUDIES IN CROP VARIATION. II. THE MANURIAL RESPONSES OF DIFFERENT POTATO VARIETIES. *Journ. Agr. Sci. [England]* 13 [311]-320 1923.

The regression of sugar percentage on weight is given by the coefficient of  $w$  in the formula

$$Z = \bar{z} + b(w - \bar{w}),$$

where  $Z$  is the estimated sugar percentage,  $\bar{z}$ , the mean sugar percentage,  $(w - \bar{w})$ , the deviation of a given weight from the mean weight; and  $b$ , the regression coefficient calculated from

$$\frac{S(zw - \bar{z}\bar{w})}{S(w - \bar{w})^2}.$$

The calculated regression coefficient for variation within plots would then be given by

$$\frac{-212.4120}{360\ 4020}, \text{ or } -0.589375,$$

and the regression equation by

$$Z = 14.0573 - 0.589375 (w - \bar{w})$$

Since the mean weight ( $\bar{w}$ ) was 1 7738 pounds, the regression equation can be expressed more conveniently by  $Z = 15\ 1027 - 0.589375 w$ , where  $w$  is any observed weight, the mean sugar percentage being 14.0573 per cent. This would then express the sugar percentage predicted on the basis of its relationship to weight. As weight increased by 1 pound, the predicted sugar percentage decreased by 0.59 per cent. The correlation between weight and sugar percentage, within plots, was  $-0.2551$ . This correlation coefficient is shown to be undoubtedly significant when a test of significance is applied, therefore the regression coefficient must be significant also. The significance of the latter will be determined later in another way.

In order to determine whether the regression of sugar percentage on yield was essentially linear or whether the quadratic regression would describe more accurately the exact relationship, the term  $w^2$  was introduced, i. e., each weight was squared and the results were considered a third variable. By calculating the variance of  $w^2$  and the covariance of  $w^2$  with  $w$  and  $z$ , using the analysis-of-variance method previously illustrated, the values given in Table 4 were obtained.

TABLE 4.—Numerical values of sums of squares or of products

Sum of squares or of products	Within plots	Between plots	Sum of squares or of products	Within plots	Between plots
$S(z - \bar{z})^2$ .....	1923 6990	384. 3977	$S(w^2 - \bar{w}^2)$ .....	5819 7071	861. 3657
$S(w - \bar{w})^2$ .....	360 4020	55 4516	$S(w^2w - w^2\bar{w})$ .....	1405 8448	210 5334
$S(zw - \bar{z}\bar{w})$ .....	-212 4120	-49 2867	$S(zw^2 - \bar{z}w^2)$ .....	-890 8212	-186 0060

times. The 5 per cent point is taken as a convenient minimum level of significance. Since the value of  $z$  is not given for  $n_1=99$  and  $n_2=900$ , it must be calculated.<sup>9</sup> The 5 per cent point is found to be at 0.1165 and the 1 per cent point at 0.1636. The observed value of  $z$  exceeds the 1 per cent point. This would indicate that there was a significantly greater variation between plots than within plots. Soil heterogeneity, therefore, was a significant factor in affecting the sugar percentage in different plots.

The analysis of variance of weight is given in Table 2. The variation in weight between plots is undoubtedly greater than the variation within plots, since the observed value of  $z$  exceeds the 1 per cent point.

TABLE 2—Analysis of variance of weight

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	$z$
Between plots.....	99	55 4516	0 5601	0 7484	} 0 1679
Within plots.....	900	360 4020	4004	6328	
Total.....	999	415 8536	4163	6452	-----

The covariance of sugar percentage and weight is given in Table 3. The covariance is found by multiplying the sugar percentage by the weight, summing, and subtracting the general sum of one variable multiplied by the general mean of the other.

TABLE 3—Analysis of covariance of weight and sugar percentage

Variation	Degrees of freedom	Sum of products	Mean product
Between plots.....	99	-49 2867	-0 4978
Within plots.....	900	-212 4120	- 2360
Total.....	999	-261 6987	- 2620

The regression of sugar percentage on weight may now be calculated and the significance and linearity of such regression determined. The regression of sugar percentage on weight within plots should yield the most exact determination available from the data. By dealing with variations within plots of one row 2 rods long, the regression will be unaffected by soil heterogeneity for both weight and sugar percentage between plots. The regression coefficient calculated from observations taken over a large area might easily be affected greatly by differential soil heterogeneity. A positive relationship might be found from data obtained from a large area and a negative relationship from data obtained from a small area. The studies of small areas would tend to give the more exact relationship, since they would be influenced much less by soil variability.

In order that the notation shall not be confusing, the symbol  $w$  will be used to designate the weight of roots in pounds,  $\bar{w}$ , the mean weight of roots,  $Z$  (Zucker), the estimated sugar percentage,  $z$ , an observed value of sugar percentage, and  $\bar{z}$ , the mean sugar percentage.

<sup>9</sup> FISHER, R. A. Op. cit., Table VI.

In testing the significance of the linear and quadratic regressions the value of  $z$  exceeded the 1 per cent point, and it may be concluded that the regressions are undoubtedly significant. In testing the increased accuracy of the quadratic over the linear regression the observed value of  $z$  (0.8788) exceeded the 5 per cent point but not the 1 per cent. About 92 per cent of the quadratic regression could be represented by the linear equation. It may be concluded, therefore, that the quadratic regression probably was a better measure of the regression of sugar percentage on weight than the linear equation. The regression was not entirely linear.

Given the variances for sugar percentage and weight between plots and the regression of sugar percentage on weight within plots, it is possible to determine whether sugar percentage varied significantly from plot to plot, even when the effect of weight on sugar percentage was held constant, on the basis of the regression relationship. If the variation in sugar percentage is significant after being so corrected it may be concluded that soil heterogeneity was such as to affect sugar percentage significantly apart from the indirect effect on sugar percentage caused by soil variability affecting weight. Such a study would shed light on the question of whether soil heterogeneity affected weight and sugar percentage independently.

#### VARIABILITY IN SUGAR PERCENTAGE BETWEEN PLOTS AFTER CORRECTION FOR REGRESSION ON WEIGHT

From the data already given, the variability in sugar percentage between the different plots, holding constant the effect of weight on sugar percentage (as expressed by the regression coefficient), may be determined. Using the linear regression, the sum of squares due to variation in sugar percentage between plots, corrected for regression of sugar on weight within plots, may be calculated from

$$S\{(z - \bar{z}) - b(w - \bar{w})\}^2$$

This formula may be expanded into

$$S\{(z - \bar{z})^2 - 2b(zw - z\bar{w}) + b^2(w - \bar{w})\}^2$$

Substituting the necessary sums of squares or products and the regression coefficient,

$$384\,3977 - 2(-0.589375)(-49\,2867) + (0.589375)^2(55\,4516) = 345\,5628$$

The analysis of variance is shown in Table 6

TABLE 6—Analysis of variance in sugar percentage between plots after correcting for linear regression of sugar percentage on weight within plots

Variation	Degrees of freedom	Sum of squares	Mean square	$z$
Between plots corrected for weight.....	99	345 5628	3 4905	} 0.2783
Deviation from linear regression.....	899	1,798 5087	2 0006	

The variance of sugar percentage between plots has been reduced only 10 per cent by correcting for the regression of sugar percentage on weight, i. e., the variance after correction is about 90 per cent of

The quadratic regression that will satisfy the equation

$$Z = \bar{z} + b(w - \bar{w}) + c(w^2 - \bar{w}^2)$$

may be found by substituting in the following simultaneous equations, and solving

$$bS(w - \bar{w})^2 + cS(w^2w - w^2\bar{w}) = S(zw - z\bar{w})$$

$$bS(w^2\bar{w} - w^2\bar{w}) + cS(w^2 - \bar{w}^2)^2 = S(zw^2 - z\bar{w}^2)$$

Substituting the appropriate values for the sums of squares or products within plots in these formulae and solving,  $b = 0.133687$  and  $c = -0.185364$ . The quadratic regression equation would then be

$$Z = 14.0573 + 0.133687(w - \bar{w}) - 0.185364(w^2 - \bar{w}^2)$$

or, expressed more suitably for calculation,

$$Z = 14.4805 + 0.133687w - 0.185364w^2$$

since  $\bar{w} = 1.7738$  and  $\bar{w}^2 = 3.5622$ .

The significance of the regression coefficient may be tested by apportioning the total sum of squares within plots to the part due to linear regression and to the part due to deviation from regression. The part due to linear regression will be given by

$$bS(zw - z\bar{w})^{10}$$

The part due to quadratic regression will be given by

$$bS(zw - z\bar{w}) + cS(zw^2 - z\bar{w}^2).$$

The analysis of variance is shown in Table 5.

TABLE 5—Tests of significance of linear and quadratic regression and of increased accuracy of quadratic over linear regression

LINEAR REGRESSION							
Variation due to—	Degrees of freedom		Sum of squares		Mean square		<i>z</i>
Linear regression.....	1	-----	125 1903	-----	125 1903	-----	} 2.0682
Deviation from linear regression.....	899	-----	1,798 5087	-----	2 0006	-----	
Total within plots.....	900	-----	1,923 6990	-----	2 3104	-----	
QUADRATIC REGRESSION							
Quadratic regression.....	2	-----	136 7295	-----	68 3648	-----	} 1.7684
Deviation from quadratic regression.....	898	-----	1,786 9695	-----	1 9899	-----	
Total within plots.....	900	-----	1,923 6990	-----	2 3104	-----	
QUADRATIC AND LINEAR REGRESSION COMPARED							
Quadratic regression.....	2	-----	136 7295	-----	68 3648	-----	} 0.8788
Linear regression.....	1	-----	125 1903	-----	125 1903	-----	
Quadratic minus linear regression.....	1	-----	11 5392	-----	11 5392	-----	
Deviation from quadratic regression.....	898	-----	1,786 9695	-----	1 9899	-----	
Total within plots.....	900	-----	1,923 6990	-----	2 3104	-----	

<sup>10</sup> FISHER, R. A. Op. cit. (See footnote 8.)

will be presented later in summary form. The data from plots 2 rods long will be given first. It must be remembered that the sampling was confined to only one row in each 4-row plot. The analyses of variance are all on a single-beet or 0 1-plot basis.

The study was made on 10 beets taken from each of 100 plots 2 rods long. Since five varieties are to be tested, there will be 20 replication series or blocks. The latter term will be used. The analysis of variance for plots 2, 4, 10, and 20 rods long is shown in Table 8.

TABLE 8—Analysis of variance in sugar percentage of beets in plots of various lengths

PLOTS 2 RODS LONG					
Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	<i>z</i>
Between blocks.....	19	150 3677	7 9141	2 8132	} 0 6397
Within blocks.....	980	2, 157 7290	2 2018	1 4838	
Total.....	999	2, 308 0967	2 3104	1 5200	
Between plots.....	80	234 0300	2 9254	1 7104	} 0 1569
Within plots *	900	1, 923 6990	2 1374	1 4620	
PLOTS 4 RODS LONG					
Between blocks.....	9	99 1730	11 0192	3 3195	} 0 7985
Within blocks.....	990	2, 208 9237	2 2312	1 4937	
Total.....	999	2, 308, 0967	2 3104	1 5200	
Between plots.....	40	159 4352	3 9859	1 9965	} 0 3069
Within plots *	950	2, 049 4885	2 1574	1 4688	
PLOTS 10 RODS LONG					
Between blocks.....	3	22 6335	7 5445	2 7467	} 0 5991
Within blocks.....	996	2, 285 4632	2 2946	1 5148	
Total.....	999	2, 308 0967	2 3104	1 5200	
Between plots.....	16	55 2002	3 4500	1 8574	} 0 2080
Within plots *	980	2, 230 2630	2 2758	1 5086	
PLOTS 20 RODS LONG					
Between blocks.....	1	8 0102	8 0102	2 8302	} 0 6229
Within blocks.....	998	2, 300 0865	2 3047	1 5181	
Total.....	999	2, 308 0967	2 3104	1 5200	
Between plots.....	8	30 3634	3 7954	1 9482	} 0 2521
Within plots *	990	2, 269 7231	2 2926	1 5141	

\* Derived by subtracting the calculated values between plots from the values within blocks

The total sum of squares may first be apportioned to that part due to variation between blocks of five varieties each and to that part due to variation within these blocks. The sum of squares within blocks may be subdivided into that part due to variation between plots within the blocks and that part due to variation between beets within individual plots. The observed *z* value (0 6397) for the 2-rod plots, since it exceeds the 1 per cent point, shows that the variance between blocks was significantly greater than the variance within

the variance between plots before correction. The observed value of  $z$  exceeds the 1 per cent point, and it may be concluded that the variation in sugar percentage between plots was quite significant even when the effect of weight was held constant. Apparently, soil differences in different plots were such as to affect significantly the sugar percentage apart from the indirect effect due to weight.

In like manner a test may be made holding constant the effect of weight on sugar percentage on the basis of the quadratic regression relationship. The sum of squares measuring the variability in sugar percentage between plots, holding constant the effect of sugar percentage on weight within plots, is then given by

$$S\{(z - \bar{z}) - b(w - \bar{w}) - c(w^2 - \bar{w}^2)\}^2$$

Expanding this as before and substituting the appropriate values, a variance of 348 7712 is obtained. Only 9 per cent of the variance between plots has now been removed by the quadratic regression. The analysis of variance is shown in Table 7. The observed value of  $z$  again exceeds the 1 per cent point, and we conclude that there was a significant variation in sugar percentage between plots, even when corrected for quadratic regression of sugar percentage on weight within plots. There can be little question, therefore, that soil heterogeneity affected both weight and sugar percentage independently to an appreciable degree.

TABLE 7 — Analysis of variance in sugar percentage between plots after correcting for quadratic regression of sugar percentage on weight within plots

Variation	Degrees of freedom	Sum of squares	Mean square	$z$
Between plots, corrected for weight.....	99	348 7712	3 5229	} 0 2856
Deviation from quadratic regression.....	898	1786 6695	1 9899	

#### SIZE OF SAMPLE IN RELATION TO DETERMINATION OF SUGAR PERCENTAGE

The analysis of variance designed to determine variability between individual beets within a plot of given size and between the means of different plots can be made most advantageously by assuming a hypothetical experiment designed to test a given number of varieties. The total sum of squares can be apportioned to that part due to variation between blocks and that part due to variation within blocks. When the arrangement of plots within each block, or replication series, is random, it will always be legitimate to eliminate the variability between blocks from the total variability in determining the part that may be ascribed to error. The assumption of a given number of varieties to be tested and the elimination of variability between blocks containing these varieties seems, therefore, a valid one. The sum of squares due to variations within blocks can be divided further into that part due to variation between the plots within a block and that due to variation within plots. The study presented here was made on the assumption that 5 or 10 varieties or treatments were to be tested. The results on the assumption of five varieties tested will be given first and in some detail. The results for 10 varieties tested

sample for a given number of replications and a given degree of accuracy can be calculated conveniently from

$$n = \frac{m}{KN - p}$$

For example: To reduce the standard error of the mean to a given level when plots 2 rods long were used and five varieties tested (see Table 8), where  $m = 2\ 1374$  and  $p = 0\ 0788$ ,

$$n = \frac{2\ 1374}{KN - 0\ 0788}$$

To obtain a standard of 0.1 (variance 0.01) with 10 replications,

$$n = \frac{2\ 1374}{(0\ 01)(10) - 0\ 0788} = 100 \text{ (approximately).}$$

It would require 100 beets per plot to obtain a standard error of 0.1, using 10 replications.

TABLE 9—Number of beets per plot, when five varieties are tested, necessary in analysis to reduce the standard error of mean sugar percentage to 0.3, 0.2, and 0.1 per cent, for various lengths of plots and numbers of replications

Number of replications	Number of beets per plot of indicated length (rods) necessary to reduce standard error of mean to—											
	0.3 per cent				0.2 per cent				0.1 per cent			
	2	4	10	20	2	4	10	20	2	4	10	20
4	8	8	7	7	26	31	17	16			138	92
6	5	5	4	4	13	15	11	10			62	51
8	3	3	3	3	9	9	8	8			40	35
10	2	3	3	3	7	7	6	6	<sup>a</sup> 100	<sup>a</sup> 251	30	27
20	1	1	1	1	3	3	3	3	18	20	13	12

<sup>a</sup> Size of sample exceeded the number of beets expected in 2 rows of the plot, with a perfect stand

In Table 9 is given the size of sample necessary to reduce the standard error of the mean to 0.3, 0.2, and 0.1 per cent sugar with various numbers of replications. The number of beets required is given in whole numbers. A standard error of the mean of 0.3 or 0.2 could be obtained easily, i. e., with a relatively small number of sugar analyses, especially if the sugar analyses were made on bulk samples from the different plots. A standard error of the mean of 0.1 could be obtained only by using much larger samples and longer plots, particularly for the smaller number of replications. In fact, it would be impossible to obtain a standard error of the mean sugar percentage of 0.1 by using plots 2 or 4 rods long and replicating only 10 times or less. For four and six replications and plots 2 or 4 rods long the variance due to inherent differences in the soil (expressed by  $p$ ) was itself greater than the desired variance ( $KN - p$  was negative), and a standard error of 0.1 could not be obtained regardless of size of sample. An accuracy of 0.1 could be obtained only from large plots or from a large number of replications of the short plots.

blocks. The variance between plots within the blocks was significantly greater than the variance within plots, since the  $z$  value (0.1569) exceeds the 5 per cent but not the 1 per cent point. It is from the variance between plots that the error of the test must be calculated. The variance within plots provides an estimate of the sampling error.

With the 4-rod plots the first value of  $z$  exceeds the 1 per cent point and the second the 5 per cent. Both may be judged significant. With the 10-rod plots the first value of  $z$  exceeds the 5 per cent point and the second does not. With the 20-rod plots neither value of  $z$  exceeds the 5 per cent point, and it can not be concluded that the variances compared were really different.

The data from the 2-rod plots in Table 9, considered critically, reveal several interesting facts. The first is that the variance between plots within blocks is due, to a great extent, to the variation within plots, i. e., the errors in sampling. The variance between plots (2 9254) is due to two components, namely, the sampling variance (2 1374) and a residue (0 7880) due to soil heterogeneity between plots, that is, 73.1 per cent of the variance between plots is due to sampling errors. The variation due to actual soil differences between plots (variance 0 7880) can be reduced only by increased replication. The sampling error (variance 2 1374) can be reduced only by increasing the size of sample. The standard error between plots of any given magnitude can then be estimated, on this basis, for various numbers of replications and sizes of sample per plot.

A table may be constructed giving the size of sample necessary to reduce the standard error of the mean sugar percentage to 0.3, 0.2, and 0.1 per cent sugar when 4, 6, 8, 10, and 20 replications are used for plots 2, 4, 10, and 20 rods long. Since the samples were taken from 1 row in each 4-row plot, such a table would give the size of sample needed when only 1 row was sampled. In practice the two central rows from each plot probably would be sampled. The variance within plots increased but slowly with increasing length of row. It is to be expected, then, that the sampling variance would not have been increased greatly had the samples been taken from the two central rows of each plot instead of from a single row. A formula giving the size of sample needed to obtain a given standard error of the mean for a given number of replications may be devised as follows:

Given:

$K$  = required variance of mean sugar percentage

$N$  = number of replications

$n$  = number of beets per plot

$m$  = variance between single beets within plots.

$p$  = variance between plots due to soil differences, i. e., variance between plots within blocks minus variance within plots, expressed on a plot basis.

Then

$$K = \frac{1}{N} \left( p + \frac{m}{n} \right)$$

will give the number of replications and size of sample necessary to reduce the variance of the mean to any given level ( $K$ ). The size of

The standard error of sugar per beet was approximated from the formula

$$\sqrt{\bar{z}^2 V(w) + \bar{w}^2 V(z) + 2 \bar{z} \bar{w} V(wz)},$$

where  $V(w)$  and  $V(z)$  are the variances of weight and sugar percentage, respectively, and  $V(wz)$  is the covariance

In Table 11 is given the standard error in percentage of the mean for the variations in weight, sugar percentage, and total sugar per beet between plots in blocks of 10 varieties

TABLE 11—Standard error per beet of weight, sugar percentage, and total sugar, expressed in percentage of the mean

Length of plot in rods	Standard error (in percentage of mean) for—		
	Weight	Sugar percentage	Sugar per beet
2.....	41 3	13 2	37 4
4.....	41 1	15 2	36 9
10.....	56 5	14 7	48 3
20.....	61 6	14 7	54 4

The individual roots varied tremendously in weight and much less in sugar percentage. The variability in the product of weight times sugar percentage, or sugar per beet, was intermediate. With no correlation, the standard error of sugar per beet would be the square root of the sum of the variances of weight and sugar percentage weighted by the squares of the mean sugar percentage and mean weight, respectively. A negative correlation would reduce this quantity, depending on its magnitude.

#### VARIATION IN SUGAR PERCENTAGE DETERMINED FROM THE MEAN OF 10 INDIVIDUAL ROOTS AND FROM BULK SAMPLES OF 10

Besides the 10 individual sugar-beet analyses made from beets on each of the 100 plots, a bulk sample of 10 other beets (taken from the same rows) was also analyzed for sugar. The entire sample was ground, the juice expressed in a hydraulic press, and the sugar percentage in the juice determined by Horne's dry lead method. The bulk sample was taken over the entire length of the row in the same manner as the individuals. A comparison was then made of the variances between plots based on the mean of 10 individual sugar analyses and on the bulk analysis of 10 beets made on the same plots. The results, given in Table 12, are on a 10-beet basis.

TABLE 12—Variability of means of 10 beets per plot analyzed individually and 10 beets analyzed as a composite sample

Variation between plots determined from—	Degrees of freedom	Mean square	Standard deviation	<i>z</i>
Means of 10 individuals.....	99	0 3883	0 6231	} 0 0604
Bulk samples of 10 individuals.....	99	2739	5234	

As replication was increased, the total number of beets needed for sugar analysis decreased. With plots 4 rods long and with 4 replications it would be necessary to analyze 124 beets to obtain a standard error of 0.2 per cent sugar. If 20 replications had been used, only 60 beets would have been required to obtain the same standard error.

In Table 10 are given the results on the basis of 10 varieties tested. Since 10 varieties would require more land per block than 5 varieties, the amount of variance between blocks that can be removed would be less. This would leave a greater variance within blocks on a 10-variety basis. Greater variability within blocks would mean increase in size of samples needed to obtain the same accuracy as could be obtained were only five varieties tested. This was particularly true for the smaller number of replications, since the variance between plots due to inherent soil differences, represented by  $p$ , was greater than for five varieties tested, and consequently played a more important rôle. The variance represented by  $m$  was the same as in the 5-variety test.

TABLE 10—Number of beets per plot, when 10 varieties are tested, necessary in analysis to reduce the standard error of mean sugar percentage to 0.3, 0.2, and 0.1 per cent, for various lengths of plots and numbers of replications

Number of replications	Number of beets per plot of indicated length (rods) necessary to reduce standard error of mean to—											
	0.3 per cent				0.2 per cent				0.1 per cent			
	2	4	10	20	2	4	10	20	2	4	10	20
4.....	9	9	7	7	<sup>a</sup> 73	53	19	16	-----	-----	<sup>a</sup> 3,793	113
6.....	5	5	6	4	20	18	11	10	-----	-----	110	57
8.....	4	4	4	3	11	11	8	8	-----	-----	56	38
10.....	3	3	3	3	9	8	6	6	-----	-----	38	29
20.....	1	1	1	1	3	3	3	3	31	27	14	13

<sup>a</sup> Size of sample exceeded the number of beets expected in 2 rows of the plot, with a perfect stand

#### MEAN AND STANDARD ERROR OF TOTAL SUGAR PER BEET

The mean sugar per beet (expressed in pounds) may be obtained by multiplying the weight per beet by the sugar percentage and averaging the quantities obtained. It may be obtained also by direct calculation from the formula

$$\bar{w} \bar{z} + \frac{S(w - \bar{w})(z - \bar{z})}{n'}$$

where  $\bar{w}$  and  $\bar{z}$  are the mean weight and sugar percentage, respectively,

$$\frac{S(w - \bar{w})(z - \bar{z})}{n'}$$

is the covariance of weight and sugar percentage, and  $n'$  is the actual number of beets. Since the mean weight was 1.7738 pounds, the mean sugar percentage (expressed as a decimal fraction) was 0.140573, and the covariance was 0.002617, the mean total sugar per beet would be 0.2467 pound.

Soil heterogeneity between plots was found to affect sugar percentages significantly even when the effect of weight was held constant by means of the regression relationship

Tables are given showing the number of beets per plot needed to reduce the standard error of the mean to 0.3, 0.2, and 0.1 per cent sugar for various sizes of plots and numbers of replications.

Variability in sugar percentage between plots and within plots must be considered in estimating the size of sample required and the number of replications needed to reduce the standard error to a given level.

The standard error of the mean of total sugar per beet was somewhat lower than the standard error for weight and much higher than that for sugar percentage

Variability in sugar percentage between plots was essentially the same whether calculated from the mean of 10 beets analyzed individually or from a composite sample of the same number

The observed value of  $z$  does not exceed the expected 5 per cent point value of 0.1628, and it may be concluded that the two variances were not significantly different. We may assume, therefore, that the conclusions based on the variability of individual sugar determinations will be a valid estimate of the results to be expected from sugar determinations based on composite samples.

### DISCUSSION

The dual effect of soil heterogeneity was illustrated in a striking manner by the demonstration that only a small portion of the variation in sugar percentage between plots within blocks was due to the same factors that affected both weight of roots and sugar percentage. A given standard error for weight or sugar percentage can be obtained only from a consideration of number of replications and size of sample. This must be determined almost independently for both.

The studies on size of sample revealed several interesting facts. Standard errors of 0.3 and 0.2 per cent sugar could be obtained fairly easily, while a standard error of 0.1 per cent could be secured only with much greater difficulty. Increasing the replication reduced the total number of beets needed for sugar analysis. This reduction was not uniform, however, as replication was increased. The greatest reduction in total number of beets needed came when the replications were increased from four to six, and decreased more and more slowly with further replication.

By knowing the cost of the field operations and the cost of the sugar analyses in the laboratory, it would be a simple matter to determine the size of plot and the number of replications that would give the required standard error at minimum cost. In practice the best size of plot and number of replications would have to be determined from variability studies for yield as well as for sugar percentage. Therefore, the cost of the yield trials in relation to the accuracy of results obtained would need to be considered also.

It seems evident that in sampling studies variability both between plots and within plots must be considered. This was shown by the fact that a standard error of the mean of 0.1 per cent in sugar could not be obtained from plots 2 or 4 rods long replicated six times or less. The error due to responses to soil differences between plots exceeded this value, making it impossible to obtain a standard error of 0.1 per cent in this study, regardless of size of sample.

In practice it would seem advisable to take at least two samples from each plot, even when composite samples are used, so that the analysis of results shall always give a measure of the sampling error. The latter would serve as a constant check on the accuracy of the sampling method. Such samples would need to be taken so that each would sample uniformly the entire plot considered, but the two sampling units should be taken at random.

### SUMMARY

Sampling technic was studied in relation to the determination of sugar percentage in sugar beets.

Regression of sugar percentage on weight of roots was not entirely linear. Ninety-two per cent of the quadratic regression could be explained in terms of the linear function.

# SIZE AND SHAPE OF PLOT IN RELATION TO FIELD EXPERIMENTS WITH SUGAR BEETS<sup>1</sup>

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## INTRODUCTION

The literature dealing with field experiments is very extensive. Studies to determine the most efficient size and shape of plot and the value of replication have been conducted with a great variety of crops and under widely diverse conditions. The committee for the standardization of field experiments of the American Society of Agronomy has given a complete bibliography dealing with this subject.<sup>3</sup>

Studies on size and shape of plot in relation to field experiments with sugar beets (*Beta vulgaris* L.) are of special interest, since they must be concerned not only with yield but also with sugar percentage, total sugar per plot, and percentage of purity. The optimum size and shape of plot for determining one of these characters is not necessarily the most efficient for the other characters. All four must be considered and their relationship to one another determined.

Pritchard<sup>4</sup> studied the value of check plots and repeated plantings in variety trials with sugar beets and concluded that frequent checks could be used to advantage in calculating the error of the experiment. He also found that the error of the experiment was reduced with increased replication. The reduction was most pronounced as replication increased to 7; smaller gains were obtained for greater replications, i. e., up to 10.

The yields of relatively small plots, such as are used in agronomic experiments, will usually be determined by harvesting the entire plot. Except for very small plots it would not seem necessary to analyze all the beets in a plot for sugar percentage. Sampling methods must be resorted to, therefore, in selecting beets for sugar analysis. This introduces another error, the error in sampling. The writer has studied sampling technic with sugar beets in relation to the determination of sugar percentage of the roots and has discussed the problem in some detail.<sup>5</sup> The present study was made from data obtained from the same field as was used for the study of sampling technic. The data on sugar percentage<sup>6</sup> and apparent purity<sup>7</sup> used in this

<sup>1</sup> Received for publication Nov 5, 1931, issued May, 1932. Contribution from the National Research Council and from the U. S. Department of Agriculture in cooperation with the Minnesota Agricultural Experiment Station.

<sup>2</sup> Fellow of the National Research Council. The writer takes great pleasure in recording his indebtedness to Dr. R. A. Fisher, chief statistician of the Rothamsted Experimental Station, Harpenden, Herts, England, under whose guidance and in whose laboratory this study was made. He also wishes to express his appreciation to Dr. J. Wishart for helpful suggestions given during the course of the study.

<sup>3</sup> AMERICAN SOCIETY OF AGRONOMY, COMMITTEE FOR THE STANDARDIZATION OF FIELD EXPERIMENTS. REPORTS. Jour. Amer. Soc. Agron. 18: 1143-1144, 1928, 22: 1058-1061, 1930.

<sup>4</sup> PRITCHARD, F. J. THE USE OF CHECKS AND REPEATED PLANTINGS IN VARIETAL TESTS. Jour. Amer. Soc. Agron. 5: 65-81, illus. 1916.

<sup>5</sup> IMMER, F. R. A STUDY OF SAMPLING TECHNIC WITH SUGAR BEETS. Jour. Agr. Research 44: 633-647.

<sup>6</sup> Sugar percentage (as used here) is the percentage of sucrose in the beet.

<sup>7</sup> Apparent purity (as used here) =  $\frac{\text{Percentage polarization} \times 100}{\text{Corrected Brix spindle reading}}$ .



ing operations were completed in  $2\frac{1}{2}$  days and the sugar percentage determinations in  $3\frac{1}{2}$  days

The "analysis of variance" method devised by Fisher was used in analyzing the data.<sup>9</sup> The principle of the method may be given very briefly. If the total variability of the observations on all the plots is given in suitable terms (sum of squares) it may legitimately be apportioned to various known causes, leaving a remaining portion ascribable to uncontrolled or unknown causes. The latter will then serve as a basis for the calculation of the error of the experiment. The variance (standard deviation squared) due to any of the known causes or to the uncontrolled or unknown causes may then be found by dividing the sum of squares by the appropriate number of degrees of freedom. The term "degrees of freedom" is here used in the sense of "independent comparisons." With  $n$  quantities whose mean is fixed there are in general  $n-1$  independent comparisons, or degrees of freedom. The data obtained in this study were analyzed by this method, and the significance of the results obtained was determined by reference to tables given by Fisher<sup>10</sup> for testing the significance of such results

## EXPERIMENTAL RESULTS

### ANALYSIS OF YIELD DATA

The analysis of variance of yield, sugar percentage, and apparent purity was made on the assumption of five varieties or treatments to be tested. If the arrangement of the varieties within each replication series, or block, is a random one, it is legitimate to remove the sum of squares attributable to variations between blocks from the total sum of squares, leaving the sum of squares due to variation between plots within blocks from which to calculate the error of the experiment. Ordinarily this will give a lower estimate of the error of the experiment than the total standard deviation. Usually an appreciable portion of the total variance can be removed in this way. The effect of increased replication can be determined easily. The standard error of the mean of several replications would be found by dividing the standard error (standard deviation) of a single plot by  $\sqrt{N}$ , where  $N$  is the number of replication series, or blocks.

The analysis of yield data will be given first. The plot yields from which these analyses were made are given in Table 1. The standard errors between plots within blocks were calculated for 24 different sizes and shapes of plots, considering the entire plot as harvested. With plots 3 or more rows wide, a single row could be removed from each side of the plot to correct for competition between varieties. The standard errors were calculated, therefore, on this basis also for plots 3 or more rows wide and the results compared with those obtained when the border rows in each plot were not removed.

<sup>9</sup> FISHER, R. A., and MACKENZIE, W. A. STUDIES IN CROP VARIATIONS. II. THE MANURIAL RESPONSE OF DIFFERENT POTATO VARIETIES. *Jour Agr Sci [England]* 13 [311]-320, 1923.

<sup>10</sup> FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed. 3 rev and enl, 283 p., illus. Edinburgh and London, 1930.

study were obtained by sampling methods but slightly different from those of the previous study. The analysis of the data will be made in the same manner as before.

#### MATERIAL AND METHODS

A small field of sugar beets of approximately nine-tenths of an acre in area, planted with Pioneer variety in 1930, was chosen for the experiment. The field had been cropped in a uniform manner for several years prior to 1930, and cultural conditions were uniform throughout the field during the growing season. The beets were planted in rows 22 inches apart on May 5. At thinning time the field was cross marked, dividing the rows into 12-inch units, and a single beet was left in each unit. While the spacing was not exactly 12 inches in all cases, slight adjustments being made between the cross marks, the number of beets left after thinning averaged very nearly one per foot of row. The plot was cultivated during the growing season with hand cultivators. At harvest time the field was marked out into plots 2 rods (33 feet) long, with 2-foot alleys between the ends of adjacent plots. The beets in these alleys were removed by hand before harvest in order to minimize errors due to the beets being dragged from one plot to another by the beet lifter. The field actually harvested, after removing border rows and the ends of the field, consisted of 60 rows 350 feet long, the rows being subdivided into 10 series of plots each 2 rods long, with 2-foot alleys between. All beets adjacent to noticeable skips in the row were removed before harvest. After correction for such skips the stand was approximately 85 per cent of a perfect stand. The beets were harvested during the first week in October.

The beets were lifted with a regular beet lifter. A sample of 10 beets was next taken from each ultimate unit (1 row 2 rods long) at uniform intervals over the entire length of the row. Approximately every third beet was taken for the sample, the exact number depending on the total number of beets in the plot after removal of beets adjacent to skips. These sample beets were topped, placed directly in labeled waterproof bags, and removed to the laboratory. There they were weighed, washed clean of dirt, and weighed again. The entire sample was then ground by a grinder of the multiple-saw type, the juice was extracted from the pulp by a hydraulic press under constant pressure, and about  $1\frac{1}{2}$  pints of juice was used for the determination of sugar percentage and apparent purity. The sugar percentage in the juice was determined<sup>8</sup> by Horne's dry lead method, and the apparent purity was determined as the ratio of sugar percentage to the corrected Brix reading.

The remaining beets in the plot were then topped and weighed. The combined weight of the beets taken for sugar samples and those from the remainder of the plot, corrected for tare, was considered the yield of the plot. The beets were counted at weighing time, and yields were calculated on the basis of 33 beets per plot of 1 row 2 rods long. The tare, as determined from the sample, was only about 5 per cent and fairly uniform from plot to plot. The harvest-

<sup>8</sup> A conversion factor of 95.2 was used as a constant to convert the percentage of sucrose in the juice to percentage of sucrose in the beet.

total), and subtracting the same product of the general total times the general mean as used in obtaining the total sum of squares. The sum of squares due to variation within blocks is the difference between the total sum of squares and that portion due to variation between blocks. Since a total of 600 plots was considered, there were 599 ( $n-1$ ) degrees of freedom attributable to the total sum of squares. There were 120 blocks (of 5 plots each) and consequently 119 degrees of freedom due to blocks,  $599-119$  (or  $4 \times 120$ ) gives 480 degrees of freedom due to variation between the 5 plots within each of the 120 blocks. The mean square or variance (standard deviation squared) is found by dividing the sum of squares by the appropriate number of degrees of freedom. The standard deviation is the square root of the mean square or variance.

TABLE 2—Analysis of variance of weight of beets in single-row plots 2 rods long

Variation—	Degrees of freedom	Sum of squares	Mean square <sup>a</sup>	Standard deviation	$z$ <sup>b</sup>
Between blocks.....	119	6,740 3456	56 6416	7 5261	} 0 4735
Within blocks.....	480	10,547 7280	21 9744	4 6877	
Total between plots.....	599	17,288 0736	28 8616	5 3723	-----

<sup>a</sup> Mean square or variance (S D <sup>2</sup>) =  $\frac{\text{Sum of squares}}{\text{Degrees of freedom}}$

<sup>b</sup>  $z$  = one-half the difference between the natural logarithms of the two variances, or the difference between the natural logarithms of the standard errors (standard deviations)

The significance of the difference between the variance between blocks and that within blocks was determined by the  $z$  test developed by Fisher <sup>11</sup>. The test consists in finding the difference between one-half the natural logarithms of the two variances, or the difference between the natural logarithms of the standard errors (standard deviations), and determining the significance of this difference by reference to tables provided by Fisher <sup>11</sup>. The value of  $z$  in these tables is given for two different levels of significance—the 5 per cent point and the 1 per cent point. When  $z$  exceeds the 5 per cent point, it is considered that a difference as great as the observed difference will be obtained less than once in 20 trials, from homogeneous material, due to the errors of random sampling. The 5 per cent point is taken as a convenient minimum level of significance. In Table 2, the observed value of  $z$  exceeds the 1 per cent point and we conclude that the difference was undoubtedly significant. Since the variance between blocks was significantly greater than the variance within, the elimination of variation between blocks has proved worthwhile. The standard error of a single row 2 rods long was, then, 4 6877 pounds or 9 16 per cent of the mean yield of 51 1880 pounds.

In like manner we may determine the standard error of 3-row plots with the outer row on each side discarded to eliminate any possible differential competition between varieties. A single row is then harvested from each 3-row plot. The analysis of variance is given in Table 3.

<sup>11</sup> FISHER, R. A. Op cit

TABLE 1—Yield of beets (pounds) from 600 single-row plots, each 2 rods long, with 22 inches between rows

Row No	Yield (pounds) from block No. —										Total yield
	1	2	3	4	5	6	7	8	9	10	
1	45 3	54 0	47 7	50 8	43 3	51 7	47 4	48 1	53 9	54 8	497 0
2	47 8	54 3	47 2	42 6	41 7	46 1	45 3	47 8	47 3	51 4	471 5
3	46 0	49 2	48 5	47 7	46 1	53 4	49 3	47 6	54 8	59 8	502 4
4	47 3	43 8	53 4	59 5	40 8	38 8	47 3	44 6	47 6	45 6	467 7
5	43 7	47 7	50 7	45 1	51 5	49 0	42 6	41 9	45 5	42 7	460 4
6	48 1	48 8	57 7	48 1	44 8	41 8	45 7	48 6	49 3	56 3	489 2
7	45 6	46 0	42 7	42 8	51 5	47 7	48 8	47 1	49 4	49 5	471 1
8	48 7	44 9	46 0	48 3	39 0	44 7	48 5	41 6	38 3	53 0	453 0
9	37 1	48 1	39 4	46 3	48 5	50 3	50 2	50 0	41 9	58 1	469 9
10	41 4	51 2	47 6	46 7	45 2	48 2	48 0	51 1	52 0	59 1	490 5
11	43 3	47 6	52 8	50 5	47 6	47 6	48 4	45 3	53 6	57 3	494 0
12	47 2	51 8	49 6	47 2	48 7	42 8	54 2	58 9	53 3	54 7	505 4
13	52 8	49 7	52 9	52 5	49 4	52 4	54 2	48 2	50 6	51 7	514 4
14	52 5	57 1	50 1	46 2	53 4	48 7	52 1	52 6	63 3	52 6	528 6
15	43 7	43 0	51 0	47 3	56 3	44 0	44 1	43 2	57 8	50 0	480 4
16	61 2	56 3	53 8	54 7	59 1	49 1	55 7	48 0	57 6	51 7	547 2
17	55 7	49 7	55 6	45 9	54 2	46 4	61 1	54 7	56 4	51 2	530 9
18	43 5	48 5	52 6	50 1	51 6	48 1	52 7	48 1	48 0	50 5	493 7
19	59 9	48 7	50 9	48 3	46 3	51 6	52 0	49 6	51 5	55 0	513 8
20	55 1	55 0	54 2	49 9	50 3	58 6	58 5	61 8	62 9	58 1	564 2
21	55 4	49 8	52 8	48 8	49 1	61 7	56 2	55 0	56 5	54 8	540 1
22	41 1	46 0	55 6	51 6	54 1	55 4	49 2	59 6	59 7	58 5	530 8
23	56 0	57 8	52 3	48 8	60 0	56 2	60 0	56 2	56 7	54 5	558 5
24	51 8	53 2	52 0	49 7	53 2	50 8	47 6	52 0	67 7	55 9	533 9
25	51 6	49 6	57 1	49 8	60 0	60 6	57 5	54 5	58 2	45 5	544 4
26	49 8	54 0	47 5	45 1	53 5	49 3	51 0	47 2	60 5	59 2	520 6
27	51 3	52 0	56 3	51 1	54 2	60 6	65 2	66 5	68 5	58 1	572 2
28	49 2	48 5	59 1	60 1	63 8	52 5	54 3	58 6	64 7	61 4	572 2
29	50 1	57 6	62 3	56 1	63 8	63 8	59 8	65 3	54 6	50 5	572 4
30	47 6	48 1	48 2	47 6	54 5	45 3	55 2	52 5	50 7	49 5	490 2
31	39 5	40 0	47 0	52 1	51 8	44 8	53 1	50 7	60 5	54 8	494 3
32	42 2	45 8	50 3	53 3	47 9	57 1	59 7	55 1	58 0	43 3	512 7
33	44 9	52 6	49 5	50 0	44 5	40 7	49 6	60 6	51 1	55 2	498 7
34	41 7	48 9	51 3	55 3	45 7	46 7	44 1	46 2	48 6	54 0	482 5
35	48 8	46 2	49 2	49 9	57 6	48 4	44 8	46 6	51 5	50 4	493 4
36	45 3	52 1	46 7	53 3	53 3	58 0	50 8	56 6	59 2	55 7	530 8
37	51 1	57 8	51 3	62 3	58 1	47 0	52 0	58 7	64 7	54 2	555 0
38	50 7	53 8	50 8	53 1	49 9	52 8	47 6	50 9	55 2	51 2	516 0
39	39 6	43 6	56 3	50 6	51 7	59 6	57 8	52 1	48 3	58 9	518 5
40	48 0	50 8	52 1	50 8	54 3	42 6	46 7	49 8	45 6	56 9	496 6
41	53 8	48 7	49 7	51 9	47 4	48 7	55 2	47 8	50 6	42 5	496 3
42	47 6	46 5	42 5	47 5	46 7	45 5	44 2	45 2	48 0	42 7	456 4
43	52 0	48 8	50 6	46 6	45 5	44 7	48 2	52 8	51 4	50 0	490 6
44	52 0	46 4	45 4	49 1	46 6	46 8	48 7	50 8	55 0	47 7	488 5
45	46 1	51 1	51 8	56 4	50 7	47 8	52 0	54 7	58 0	52 3	520 9
46	46 5	58 8	51 2	53 4	58 9	44 6	50 6	51 3	55 0	59 1	523 4
47	57 3	65 8	52 3	52 6	49 0	52 0	54 8	52 5	55 8	57 5	549 6
48	58 9	65 7	54 7	59 8	57 9	53 7	47 6	52 3	54 2	53 2	558 0
49	42 0	47 8	47 3	58 1	47 4	46 2	46 5	51 7	45 3	46 6	478 9
50	45 7	47 5	42 0	43 7	42 0	44 1	40 1	48 1	43 0	46 5	442 7
51	52 6	53 0	55 0	53 5	52 2	56 2	46 7	56 2	54 0	53 7	534 0
52	41 4	46 1	44 5	49 8	55 7	66 7	63 2	47 5	49 5	46 4	510 8
53	42 3	43 7	46 7	51 7	50 5	52 7	55 5	19 0	41 6	48 6	482 3
54	40 6	47 5	45 3	49 6	55 0	51 1	50 2	51 5	53 4	48 2	495 4
55	52 3	48 6	49 3	57 6	54 0	49 3	46 6	52 0	56 3	50 8	516 8
56	50 9	49 1	51 3	52 3	50 7	54 2	56 8	55 2	53 1	52 5	526 1
57	55 8	55 6	51 7	55 0	52 7	53 9	55 4	56 6	52 5	55 2	542 4
58	56 6	52 1	46 2	53 3	53 2	52 3	54 2	46 7	55 2	44 2	514 0
59	45 5	50 9	54 6	49 9	55 8	56 5	58 4	47 0	55 0	49 0	522 6
60	55 5	47 3	58 8	63 0	61 9	65 7	60 3	57 1	51 2	52 3	575 0
Total	2,918 0	3,024 9	3,043 0	3,067 2	3,070 6	3,048 0	3,098 7	3,087 4	3,218 4	3,136 6	30,712 8

In Table 2 the analysis of variance is given for weight of beets from plots of one row 2 rods long

The total sum of squares was obtained by squaring the weight of each plot, summing, and subtracting the product of the general total times the general mean. The sum of squares between blocks was obtained by squaring the total weight of each of the 120 blocks, summing, dividing by 5 (the number of elements contributing to each

The data from entire plots harvested will be considered first. In general the standard error, in percentage of the mean, decreased with increased size of plot, which was to be expected. Increasing the width of the plots from one row to two resulted in a very pronounced reduction in the standard error. Further increase in width of plot resulted in but slightly increased accuracy until the 12-row plots were reached. The standard errors for 6-row plots of varying length were greater than for 4-row or 3-row plots and even greater than for 2-row plots for the 10-rod and 20-rod lengths. Soil heterogeneity on this field apparently was of such a nature that 6-row plots were an undesirable width. That the fertility contour lines of the field were such as to render 6-row plots undesirable will be shown later.

Increasing the length of rows from 2 to 4 rods resulted in greatly reduced standard errors. Further increase in length of plots to 10 rods reduced the error further, but not in proportion to the greater area of land used. Still further increase in length of plot to 20 rods resulted in but slightly reduced standard errors and not at all in proportion to the area of land required. In the 4-row plots the standard error was greater in plots 20 rods long than in plots 10 rods long.

Harvesting only the central row or rows from plots 3, 4, 6, and 12 rows wide gave standard errors greater than when the entire plots were harvested, which was to be expected. In the case of 3-row plots only one-third of the entire plot would be harvested, in the 4-row plots, one-half would be harvested, etc. Increasing the length of plot reduced the standard error in essentially the same ratio as when the entire plot was harvested. Increasing the width of plot and discarding border rows reduced the standard error more rapidly than when the entire plot was harvested, because of the fact that the percentage of the plot actually harvested increased with the use of wider plots. It is to be expected, then, that when border rows are discarded plots of certain widths will prove to be more efficient in their use of the land than plots of other widths, and the most efficient plot will not necessarily be the narrowest.

In Table 5 is given the number of replications needed to reduce the standard error of the mean to 2 per cent. The standard error of the mean of several replications is found by dividing the standard error of a single plot by the square root of  $N$ , when  $N$  is the number of replications.

TABLE 5—Theoretical number of replications needed to reduce the standard error of the mean to 2 per cent

ENTIRE PLOT HARVESTED						
Length of plot	Number of replications for plots of indicated number of rows					
	1	2	3	4	6	12
Rods						
2 ----	21 0	11 5	9 0	9 8	10 0	6 4
4 ----	13 8	8 4	7 2	7 5	8 3	3 0
10 ----	8 4	5 6	4 9	4 0	6 9	3 7
20 ----	6 0	4 3	3 2	5 2	6 0	2 6
CENTRAL ROWS HARVESTED						
2 ----	-----	-----	22 7	16 6	13 5	6 7
4 ----	-----	-----	16 6	7 5	10 5	5 5
10 ----	-----	-----	9 6	5 5	9 2	4 1
20 ----	-----	-----	8 1	4 7	8 3	3 8

TABLE 3—Analysis of variance of yield of beets in 3-row plots 2 rods long, of which only the central row was harvested

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	<i>z</i>
Between blocks.....	39	1, 919 0456	49 2063	7 0147	} 0 3085
Within blocks.....	160	3, 767 2520	23 5453	4 8523	
Total.....	199	5, 686 2976	28 5744	5 3455	-----

There are now only 199 degrees of freedom attributable to total variation, since there are two hundred 3-row plots in the entire field of 600 single rows 2 rods long. Each block would require 15 rows. There would be 40 such blocks in the field and these would contribute 39 degrees of freedom, leaving 160 degrees of freedom attributable to variation between plots within blocks.

The observed value of *z* exceeded the 1 per cent point and it can be concluded that the variance between blocks was undoubtedly greater than the variance within blocks. The standard error of a single plot was here 4 8523 pounds or 9 53 per cent of the mean yield of all the central rows in the 3-row plots in the field (50 9215 pounds). The standard error was slightly greater than that found in Table 2 because of the fact that the size of the blocks had been increased threefold, allowing a smaller proportion of the total variability to be attributed to variation between blocks.

The 600 small plots in the field (Table 1) could be combined in various ways to form plots of varying size and shape. On the basis of 5 plots per block, it is possible to consider hypothetical plots 1, 2, 3, 4, 6, and 12 rows wide and 2, 4, 10, and 20 rods long. Plots of 3 or more rows each could be harvested entirely, or the central row or rows alone could be harvested, discarding one border row on each side of the plot. Using these combinations, the entire field is considered each time in studying the variance between plots. In Table 4 is given the standard error in percentage of the mean for these combinations.

TABLE 4—Standard errors, in percentage of the mean, of yields of plots varying in size and shape

ENTIRE PLOT HARVESTED						
Length of plot	Standard deviation of yields (per cent) for plots of indicated width (rows)					
	1	2	3	4	6	12
<i>Rods</i>						
2.....	9 16	6 77	6 00	6 27	6 33	5 05
4.....	7 42	5 79	5 38	5 49	5 75	3 48
10.....	5 79	4 72	4 42	4 01	5 24	3 86
20.....	4 89	4 14	3 60	4 55	4 90	3 24
CENTRAL ROWS HARVESTED						
2.....			9 53	8 15	7 34	5 18
4.....			8 15	5 48	6 48	4 68
10.....			6 21	4 71	6 08	4 07
20.....			5 70	4 33	5 77	3 92

plots 2 rods long. Dividing the variance of single-row plots 2 rods long, 21.9744 (Table 2) by 41 2244, we find that the 4-row plots were 53.3 per cent as efficient as single-row plots.

TABLE 6.—Percentage efficiency in use of land of plots varying in size and shape

ENTIRE PLOT HARVESTED						
Length of plot	Percentage efficiency of plot of indicated width (rows)					
	1	2	3	4	6	12
<i>Rods</i>						
2 ----	100 0	88 0	77 7	53 3	34 9	27 4
4 ----	76 2	62 5	48 2	35 2	21 2	28 8
10 ----	50 0	37 6	28 6	26 1	10 2	9 4
20 ----	35 1	24 5	21 6	10 1	5 8	6 7
CENTRAL ROWS HARVESTED						
2 ----			31 0	31 9	25 9	26 2
4 ----			21 2	35 3	16 6	16 1
10 ----			14 6	19 1	7 5	8 5
20 ----			8 7	11 3	4 2	4 6

Considering the entire plots harvested, the efficiency in use of land is seen to decrease with increased size of plot. While the standard errors, given in Table 4, decreased in general as the size of plot increased, the reduction was not proportional to the increased size of plot and the result was a reduced efficiency of the larger plots.

The most economical size of plot must then be determined from a consideration of the relative cost of planting, cultivating, and harvesting the larger total area needed for large plots, compared with the increased cost of planting and harvesting larger numbers of small plots in order to obtain the same standard error. For example, 4-row plots 2, 4, and 10 rods long utilized the land approximately one-half as efficiently as single-row plots. If the cost of planting, cultivating, and harvesting the 4-row plots did not exceed the cost of planting, growing, and harvesting one-half that area devoted to single-row plots, it would be more economical to use the 4-row width. If the reverse were true, the single-row plots would be more economical. In general, plots of 6 and 12 rows or plots 10 and 20 rods long would not seem economical on this basis. The increased cost due to devoting more land to the larger plots would probably be greater than the slightly increased cost of planting and harvesting slightly larger numbers of smaller plots to obtain the same standard error of the test.

The efficiency of varying sizes and shapes of plots when the border rows were removed was of even greater interest. It is seen that the 4-row plots were the most efficient in use of the land. There would, therefore, be no advantage in using 3-row plots. A greater area of land would need to be devoted to 3-row than to 4-row plots to obtain the same accuracy in the error determinations. Moreover, greater numbers of 3-row plots would have to be planted and harvested. Under average conditions the increased cost of devoting more land to 6-row and 12-row plots would probably not be compensated for completely.

Table 5 brings out in slightly different form the same features apparent from a consideration of Table 4. With a standard error of the mean of 2 per cent the standard error of a difference would be 2 times  $\sqrt{2}$ , or 2.83 per cent. Adopting twice the standard error of a difference as a convenient minimum level of significance, a difference exceeding 5.66 per cent could be considered significant with the replication numbers given in Table 5. With 4-row plots, of which only the two central rows were harvested, such accuracy could be attained by replicating the 2-row plots 17 times and the 4-row plots about 10 times. The theoretical number of replications (7.5) required for the latter size, as given in Table 5, would seem rather too low considering the values found for 4-row plots of other lengths. Ten replications would seem to be more nearly the correct number.

In the analyses of variance leading to the standard errors given in Table 4, the varieties within blocks were considered as side by side. With 6-row and 12-row plots, other arrangements within blocks might be considered also. Three varieties might be grown side by side and the other two end to end with two of the former varieties.

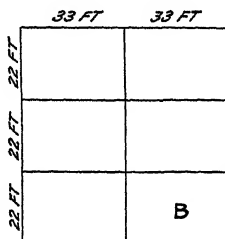
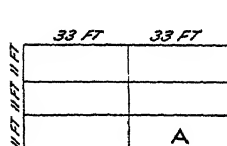


FIGURE 1.—A, Block of beets with five varieties planted in five 6-row plots, each 33 by 11 feet, B, block of beets with five varieties planted in five 12-row plots, each 33 by 22 feet

Six-row plots would then give a block of the shape shown in Figure 1, A. Twelve-row plots would give a block of the shape shown in Figure 1, B.

The arrangement of the plots within these blocks was assumed to be random. For plots 2 rods long, the standard errors in

percentage of the mean were found to be 5.46 per cent for the 6-row plots so arranged and 4.87 per cent for the 12-row plots. These were somewhat lower than the standard errors of 6.33 and 5.05 per cent, respectively, found when the five plots per block were side by side. In general it is to be expected that the more compact the block the greater will be the variation removable as variation between blocks, and the lower the standard error within blocks.

In Table 6 is given the efficiency of plots of varying size and shape calculated on the basis of variance per unit area of land. Plots 2 rows wide will require twice as much land as will plots 1 row wide. Plots 3, 4, 6, and 12 rows wide will require a corresponding number of times as much land, respectively, as will single-row plots. The efficiency of plots of different sizes and shapes in their use of the land can then be found by multiplying the variance per plot by the number of single rows 2 rods long which go to make up the plot and expressing the variance of a single row 2 rods long in percentage of these variances. Taking the variance of single rows 2 rods long as a standard, we may determine the efficiency of all other plots in relation to the efficiency of this ultimate unit of size. For example, the variance of 4-row plots 2 rods long (harvested entirely) was 10.3061. Since this is the variance of the mean of four rows, in the 4-row plots, we multiply by 4 and obtain 41.2244 as the variance of a single row in 4-row

There were five plots per block as before. The actual data from which these analyses were made are given in Table 8.

TABLE 7.—Analysis of variance of sugar percentage from 4-row plots 2 rods long, of which only the central two rows were harvested

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	<i>z</i>
Between blocks	29	36 5367	1 2599	1 1225	0.7931
Within blocks	120	30 9512	2579	5078	
Total	149	67 4879	4529	6730	

TABLE 8.—Sugar percentage of a 10-beet sample taken from each of 600 single-row plots 2 rods long, with 22 inches between rows

Row No	Sugar percentage of 10-beet sample from block No—										Total
	1	2	3	4	5	6	7	8	9	10	
1	14 60	14 41	13 85	14 24	14 22	13 18	13 66	14 02	14 14	14 58	140 90
2	14 22	14 58	14 29	13 61	13 66	14 20	13 76	14 17	14 07	14 22	140 78
3	14 31	14 52	14 99	14 09	13 76	14 80	13 90	13 81	14 51	14 36	143 05
4	14 58	14 19	14 85	13 68	13 87	13 90	13 97	13 51	14 41	14 10	140 56
5	14 14	13 66	14 40	14 17	13 83	13 20	14 22	14 34	14 36	14 63	140 95
6	14 84	13 87	14 41	13 74	14 41	13 69	13 68	14 26	15 19	14 52	142 61
7	15 38	14 14	13 44	13 95	13 35	13 71	13 20	14 43	15 87	14 60	142 07
8	14 82	14 09	14 38	14 64	14 29	14 27	13 97	14 58	14 24	14 16	143 44
9	15 87	13 37	13 66	13 66	13 98	14 58	13 90	13 51	14 48	15 65	142 66
10	13 74	13 90	14 24	13 76	13 68	13 95	13 71	13 53	14 31	15 04	139 86
11	13 88	13 29	14 87	14 48	13 71	13 27	14 27	14 29	15 50	14 87	142 43
12	14 46	14 31	14 17	14 02	14 16	14 07	14 31	14 14	14 60	15 38	143 62
13	13 81	14 48	13 51	14 82	13 49	14 14	14 05	13 29	14 38	14 90	140 87
14	14 77	14 60	14 34	13 95	14 07	14 00	14 25	14 24	15 02	14 02	143 26
15	14 07	14 26	14 78	13 81	14 35	14 22	13 95	14 36	14 41	13 61	141 82
16	14 76	14 03	13 44	14 22	15 47	13 95	13 90	14 58	14 85	14 92	144 12
17	14 76	13 52	14 00	14 31	14 65	14 43	14 14	14 79	13 71	15 06	143 37
18	13 61	13 41	14 27	13 74	14 80	13 79	14 48	14 55	14 02	14 29	140 86
19	14 31	13 60	14 55	14 00	14 63	14 09	13 78	14 05	14 12	14 58	141 77
20	14 16	15 04	14 17	13 88	13 93	13 97	14 75	14 92	14 51	14 66	143 99
21	13 90	13 44	13 95	14 51	14 58	14 55	14 00	14 51	14 60	14 38	142 42
22	14 73	14 05	14 24	14 94	15 61	14 71	14 35	14 82	14 84	14 84	147 13
23	14 00	13 97	14 43	14 65	13 95	14 24	13 43	14 24	13 92	14 60	141 43
24	14 53	14 25	13 74	14 19	14 77	13 71	15 19	14 16	14 12	14 52	143 18
25	13 64	13 61	15 36	14 09	14 22	14 41	13 83	14 38	14 15	14 82	142 51
26	14 17	13 57	14 21	14 87	14 74	14 45	14 31	14 85	13 73	15 02	143 92
27	14 48	13 15	14 82	14 97	14 12	14 71	14 02	14 70	13 93	15 19	144 09
28	14 20	14 26	14 00	14 21	13 88	14 27	14 12	15 19	15 20	14 22	143 55
29	13 78	13 99	14 12	14 48	14 58	14 09	14 43	14 07	14 70	14 68	142 92
30	13 80	14 52	15 43	14 70	14 32	14 31	14 60	13 83	14 75	15 84	146 10
31	13 90	14 87	15 01	14 82	15 40	14 80	14 00	14 51	14 82	15 41	147 54
32	14 31	14 28	14 24	14 68	14 14	14 77	14 58	14 31	14 68	15 75	145 74
33	14 17	15 00	15 09	14 55	14 09	14 84	15 24	13 88	14 87	15 07	146 80
34	15 28	14 71	15 21	15 24	15 47	14 60	14 40	14 21	15 17	15 30	149 59
35	14 05	14 43	14 58	14 84	14 75	13 90	14 73	14 45	14 82	15 28	146 43
36	14 55	15 17	14 24	14 53	14 16	14 97	14 48	14 58	15 94	14 28	146 90
37	14 07	15 28	15 94	14 48	14 48	15 35	14 79	14 16	14 63	15 90	149 08
38	14 61	14 48	15 45	14 80	14 55	14 84	14 27	14 53	14 51	15 26	147 30
39	15 40	15 17	15 04	14 31	14 67	13 78	14 27	14 24	15 87	15 63	148 68
40	14 46	14 38	14 97	14 38	14 46	14 66	14 67	15 22	15 00	14 14	145 74
41	14 46	14 84	14 63	14 48	14 61	15 04	14 63	15 01	15 50	14 96	148 16
42	13 90	14 29	14 50	14 53	15 04	15 04	15 33	14 31	15 07	14 53	146 54
43	14 00	14 84	14 50	13 95	15 04	14 94	13 92	13 73	14 07	14 35	145 24
44	14 58	14 19	14 48	14 70	14 12	14 15	14 14	14 05	15 31	15 29	145 02
45	14 43	14 80	14 09	14 61	14 48	14 83	14 65	14 92	14 27	16 23	147 31
46	14 80	14 31	14 80	14 38	14 09	14 98	15 45	13 90	14 80	14 91	146 02
47	14 41	14 92	14 21	14 73	14 46	14 92	14 38	14 63	14 99	15 60	147 26
48	14 90	14 43	14 80	14 63	14 80	14 37	14 99	14 55	14 55	15 87	147 89
49	14 55	14 05	15 15	14 80	14 53	14 70	14 48	15 82	14 89	14 85	147 67
50	15 14	14 60	14 68	14 68	14 66	15 06	15 47	15 00	14 76	15 82	150 11
51	15 47	14 63	14 22	13 76	14 82	15 07	14 71	14 92	14 66	15 16	147 42
52	14 92	14 48	14 27	15 01	15 11	15 07	15 19	14 82	15 04	16 18	150 28
53	14 55	14 27	15 10	14 82	15 06	14 80	15 04	14 92	15 19	15 34	149 09
54	14 63	15 14	13 83	14 58	15 09	14 81	14 73	14 87	15 38	15 41	148 47
55	14 63	14 75	14 99	14 48	14 34	15 07	15 01	14 58	15 35	14 84	148 04
56	15 01	15 41	14 24	15 14	14 92	15 09	15 04	14 60	15 26	15 18	149 89
57	13 86	14 99	14 97	14 50	16 27	15 37	15 06	14 17	15 19	16 20	150 58
58	14 14	15 00	14 85	14 31	14 94	14 29	15 28	15 07	14 52	15 49	147 89
59	15 51	14 70	14 80	14 46	14 24	14 89	14 60	14 58	15 24	15 89	148 91
60	15 31	14 53	13 95	14 08	15 36	14 97	14 73	15 31	14 70	16 26	150 10
Total	868 62	861 08	869 93	864 78	868 78	866 45	863 79	865 97	883 69	900 84	8,713 93

by the slightly decreased cost of harvesting a smaller number of 6-row and 12-row plots as compared with 4-row plots. The standard errors for 6-row plots were slightly higher than for 4-row plots. A probable explanation of this will be given later. In general, it is to be expected that the standard error per plot will decrease to some extent with increased size. It would seem from these data that when border rows are removed 4 rows would be the preferable width of plot and the length either 2 or 4 rods.

In order to provide a graphic illustration of the effect of soil heterogeneity on yield, the contour map shown in Figure 2 was constructed. The original yield data given in Table 1 were combined to form 6-row plots 2 rods long. The field was then considered as consisting of 100 such plots. Assuming the average yield of each plot to be at the center, the points at which yields were 5, 10, and 15 per cent above the mean and 5 and 10 per cent below the mean were found by interpolation between adjacent plots. The points found in this way for 90, 95, 100, 105, 110, and 115 per cent of the mean yield of all the

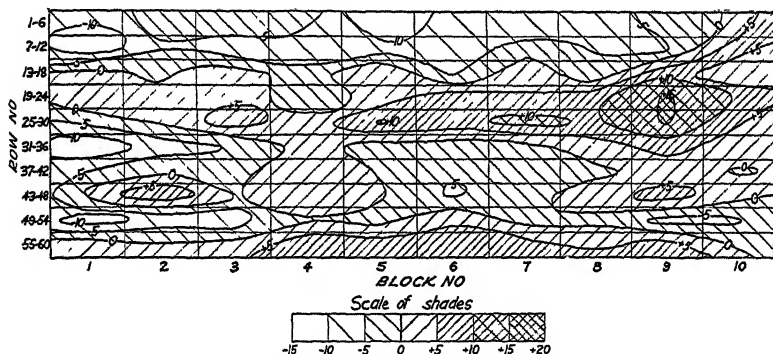


FIGURE 2—Contour map of weight of beets from one hundred 6-row plots, each 2 rods long, contour lines drawn through points deviating by  $-10$ ,  $-5$ ,  $0$ ,  $+5$ ,  $+10$ , and  $+15$  per cent from the mean weight

plots were then joined and the contour map shown in Figure 2 was constructed.

It is quite apparent that the yield varied greatly between different plots in the field. That this heterogeneity was systematic to a considerable extent is also evident. The fertility contour lines were parallel to the rows to a very pronounced degree. The latter fact probably accounts for the high error due to using 6-row plots as compared with other widths, especially when the plots were 4 or more rods long. Other plot widths did not coincide so closely with the inherent soil differences and resulted in lower standard errors. If the rows had been planted at right angles to the direction actually used, the standard errors between plots would have been reduced materially. The direction of these fertility contour lines could not be determined, however, until after harvest.

#### ANALYSIS OF SUGAR-PERCENTAGE DATA

Since the 4-row plots seemed of greatest interest, particularly when the border rows were removed, the standard errors for sugar percentage and apparent purity were calculated for this width of plot alone. In Table 7 is given the analysis of variance of sugar percentage for 4-row plots 2 rods long with only the central two rows harvested.

means of 4-row plots (only one row sampled) was 0.2925, the variance within plots (single rows) was 0.2137, and the total variance within blocks 0.2202. The data from the present study (Table 9) compared quite favorably with those results, considering the difference in area covered by the experiment, as well as the other modifications. The variance obtained in the present study from bulk analyses on 10 beets when these were ground up entirely was slightly lower than the average of 10 single beet analyses from borings through the center of the beets. It would seem, then, that the studies on size of sample made previously probably gave a conservative estimate of size of sample needed to reduce the standard error of the mean to a given level.

In Figure 3 is given the sugar-percentage contour map of the plots considered in Figure 2. The contour lines were drawn through the points where the sugar percentages were 96, 98, 100, 102, 104, and 106 per cent of the mean sugar percentage of all the plots. The points used in drawing the lines were found by direct interpola-

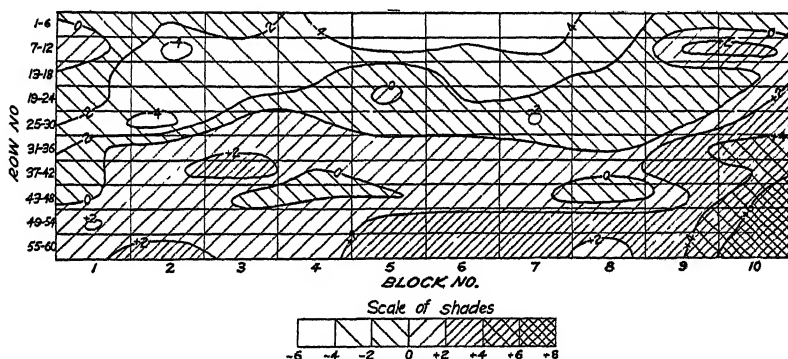


FIGURE 3—Contour map of sugar percentage of beets from one hundred 6-row plots, each 2 rods long, contour lines drawn through points deviating by  $-4$ ,  $-2$ ,  $0$ ,  $+2$ ,  $+4$ , and  $+6$  per cent from the mean sugar percentage

tion, as described for the yield contours in Figure 2. The original data are given in Table 8.

These contour lines also ran parallel to the direction of the rows, as found for yield, to a pronounced degree but not to the same extent, especially toward the right-hand side of the field. The sugar-percentage contours did not parallel the yields to an appreciable degree. The only similarity lay in the general tendency for plots giving high yields to have slightly higher sugar percentages as well, and vice versa. The actual regression of sugar percentages on yield and the tendency for soil heterogeneity to affect yield and sugar percentage independently will be given later.

#### ANALYSIS OF APPARENT-PURITY DATA

The analysis of apparent purity (expressed in per cent) for plots 4 rows wide, with only the central two rows harvested, can be made in a manner identical with that used for sugar percentage. The actual data from which these analyses were made are given in Table 10. Such an analysis of variance for plots 2 rods long is given in Table 11.

The observed value of  $z$  exceeds the 1 per cent point, indicating that a significant gain has resulted from eliminating the variability between blocks. The standard error between plots within blocks was 0.5078, or 3.50 per cent of the mean sugar percentage (14.5154), on the basis of a single 10-beet sample per plot.

In like manner the standard error between plots within blocks for similar 4-row plots 4, 10, and 20 rods long, on the basis of a single 10-beet sample per 2 rods of plot, was found to be 0.3971, 0.2356, and 0.2118, respectively. This would indicate that the variability in sugar percentage was reduced considerably by the increased size of sample from the longer plots. The standard error of sugar percentage within plots would be influenced by the size of the sample taken. The standard error of the mean sugar percentage between plots could be reduced by both replication and size of sample per plot. Both must be considered in deducing the total number of beets per plot necessary for sugar determinations and the number of replications needed in order to reduce the error to a given level. A more complete discussion of this has been given previously.<sup>12</sup> An approximation to the sampling error may be obtained from the variance between the two rows sampled in each 4-row plot. Such an analysis of variance is given in Table 9 for plots 2 rods long.

TABLE 9—Sampling error of sugar percentage from plots 4 rows wide and 2 rods long, of which only the central two rows were harvested

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	$z$
Between plots within blocks.....	120	30.9512	0.2579	0.5078	} 0.2424
Within plots.....	150	23.8215	0.1588	0.3985	
Total within blocks.....	270	54.7727	0.2029	0.4504	-----

$z$  was greater than the 1 per cent point, indicating that the variance between plots was significantly greater than the variance between the two rows within the plots. In so far as the variance between two samples from adjacent rows within the plots gives the same result as would be obtained by taking two 10-beet samples uniformly over both rows, the results may be taken as a measure of the sampling variance within plots. The variance between rows within plots on this basis could be reduced in direct proportion to the size of sample. The difference between variance between plots and within plots ( $0.2579 - 0.1588 = 0.0990$ ) would measure the response due to inherent soil differences between plots and could be reduced by increased replication alone. Sixty-two per cent ( $0.1588 - 0.2579$ ) of the variance between plots, therefore, was due to sampling error. A study was made previously<sup>13</sup> with individual sugar analyses on 10 beets taken from each 2-rod plot from rows 14, 18, 22, 26, 30, 34, 38, 42, 46, and 50. (Table 8.) The present study covered a greater area and was made on bulk analyses of 10 beets instead of 10 individual analyses on as many beets. A comparison of the results might be of interest, however. In the study on individual beets the variance between the

<sup>12</sup> IMMER, F. R. Op cit (Footnote 5)

<sup>13</sup> IMMER, F. R. Op cit (Footnote 5.)

The observed value of  $z$  exceeded the 1 per cent point and was undoubtedly significant. The standard error between plots within blocks was 2.1771, or 2.56 per cent of the mean apparent purity (84.9815), on the basis of a single 10-beet sample per plot. This is somewhat lower than the standard error calculated from the sugar percentages (3.50 per cent) on the same plots.

The standard errors between plots within blocks for plots 4, 10, and 20 rods long, on the basis of a 10-beet sample taken from each plot 2 rods long, were found by similar analyses of variance to be 1.6651, 1.0971, and 0.7875 per cent, respectively. These variances decreased markedly with increasing size of sample from the longer plots.

A direct comparison may now be given of the standard errors within blocks for yield, sugar percentage, and apparent purity for 4-row plots 2, 4, 10, and 20 rods long when only the central rows were harvested. The yields were obtained from all the beets harvested, and the sugar and purity percentages on the basis of a 10-beet sample per 2-rod plot. Expressing these in percentage of the mean we obtain the results given in Table 12.

TABLE 12—Standard errors, in percentage of the mean, of yield, sugar percentage, and apparent purity for 4-row plots of four lengths, of which only the central rows were harvested

Length of plot	Standard error of—		
	Yield <sup>a</sup>	Sugar percent- age <sup>b</sup>	Apparent purity <sup>b</sup>
Rods	Per cent	Per cent	Per cent
2-----	8.15	3.50	2.57
4-----	5.49	2.74	1.96
10-----	4.71	1.62	1.29
20-----	4.33	1.46	.93

<sup>a</sup> Calculated from total number of beets harvested on plot.

<sup>b</sup> Calculated on basis of a 10-beet sample per 2-rod plot.

Apparently weight was more variable than either sugar percentage or apparent purity, even when the latter was obtained from a 10-beet sample per plot and the former from the entire plot. The standard errors for sugar percentage and apparent purity were reduced in almost direct proportion to the increased size of sample taken from the longer plots and were not greatly affected by sampling over greater areas.

An approximation to the sampling error for apparent purity may be obtained in the manner suggested for sugar percentage (Table 9). Such an analysis of variance is given in Table 13.

TABLE 13—Sampling error of apparent purity from 4-row plots 2 rods long, of which only the central rows were harvested

Variation	Degrees of free- dom	Sum of squares	Mean square	Standard deviation	$z$
Between plots within blocks.....	120	568.7860	4.7399	2.1771	} 0.1507
Within plots.....	150	525.9800	3.5064	1.3725	
Total within blocks.....	270	1,094.7460	4.0546	2.0136	-----

TABLE 10 — Apparent purity percentage of a 10-beet sample taken from each of 600 single-row plots 2 rods long, with 22 inches between rows

Row No	Apparent purity percentage of 10 beet sample from block No —										Total
	1	2	3	4	5	6	7	8	9	10	
1	85.4	84.8	81.0	84.8	83.6	81.9	82.3	85.5	81.2	86.8	840.3
2	84.6	84.8	86.1	82.0	82.8	87.6	83.4	84.8	85.4	81.6	816.5
3	84.7	83.0	85.7	81.4	83.9	86.0	84.2	82.2	85.1	85.5	812.0
4	84.3	83.0	88.4	82.9	81.5	84.8	85.2	80.4	85.8	83.9	840.2
5	86.5	83.8	84.2	84.4	82.3	83.0	87.2	84.8	85.0	86.1	847.3
6	86.3	81.1	85.8	80.8	86.3	86.1	83.9	84.4	88.3	84.9	847.9
7	86.4	84.2	80.5	84.0	81.4	84.6	83.0	85.9	88.2	84.4	843.6
8	84.7	82.4	84.1	84.6	85.1	84.9	85.2	85.8	83.3	82.3	842.1
9	89.7	79.6	80.4	80.8	82.2	85.3	81.8	80.9	85.7	87.4	833.8
10	80.8	82.7	85.8	81.9	82.9	83.0	85.7	79.6	85.2	87.0	834.5
11	81.7	77.7	86.0	84.7	81.1	79.0	84.9	84.6	85.1	87.9	835.8
12	85.6	84.2	83.9	86.5	83.3	85.3	85.2	83.7	85.4	88.2	851.0
13	84.2	86.2	79.9	86.2	82.8	81.7	83.6	80.8	85.1	81.0	838.7
14	85.4	84.4	85.4	83.0	83.8	83.3	82.4	84.3	83.9	81.0	845.9
15	82.8	82.4	88.5	82.2	83.4	84.1	86.5	86.0	85.8	80.1	837.8
16	87.3	84.0	80.5	84.1	86.9	83.9	84.7	84.8	87.9	85.8	848.0
17	86.8	84.0	83.8	83.7	84.7	84.9	84.7	87.5	87.9	86.1	846.9
18	79.9	79.8	84.4	83.8	84.6	82.6	83.2	82.2	81.0	79.8	821.3
19	84.2	83.8	87.1	84.8	88.1	81.9	83.0	86.2	84.0	81.3	848.0
20	82.8	87.4	84.8	85.2	83.4	82.2	85.8	87.8	86.4	86.2	852.0
21	83.7	83.0	84.6	86.4	85.8	85.6	83.6	85.9	84.0	81.2	814.4
22	86.6	84.1	82.3	87.2	80.2	87.0	83.4	85.7	86.8	82.9	856.9
23	83.3	85.7	83.9	84.2	82.5	83.3	79.9	84.3	87.0	81.9	839.0
24	86.5	86.9	83.8	81.6	84.4	84.1	85.8	84.8	85.1	84.9	847.4
25	84.7	81.5	89.3	82.9	87.2	84.8	82.8	85.6	86.3	81.2	846.3
26	83.8	80.2	83.1	85.5	83.3	84.0	85.7	87.4	84.2	87.8	848.6
27	85.7	86.7	86.7	87.5	83.6	86.0	87.1	85.5	87.6	87.3	857.2
28	85.7	83.4	83.3	83.1	83.1	86.5	84.6	85.8	88.9	85.7	849.9
29	85.1	84.3	83.1	83.7	83.8	82.4	84.9	83.8	84.5	85.4	841.0
30	80.7	83.9	86.7	85.5	81.2	84.2	83.0	84.8	85.3	88.5	847.7
31	84.8	86.4	87.3	84.7	87.0	87.6	83.3	86.4	86.7	87.1	861.3
32	84.7	84.0	82.3	84.9	82.7	83.9	84.8	83.7	86.0	87.5	845.4
33	85.4	83.8	84.8	86.1	81.9	86.3	87.1	83.1	87.5	87.1	858.1
34	85.8	87.0	86.9	87.0	86.4	84.9	84.7	83.6	82.9	85.5	855.3
35	86.2	84.9	85.3	85.3	85.8	84.8	86.1	82.6	86.2	86.3	853.5
36	84.1	87.2	79.6	86.5	81.4	85.1	83.2	83.3	89.6	81.6	841.6
37	86.8	85.8	85.2	83.2	85.2	85.8	84.5	81.4	83.6	87.4	848.9
38	86.4	83.7	86.8	86.6	84.1	86.8	84.4	86.5	85.4	86.7	857.4
39	87.0	84.3	83.6	83.7	82.9	80.1	83.9	82.3	89.2	87.5	844.5
40	85.1	85.6	85.5	85.6	85.6	86.2	85.3	87.2	88.2	83.2	857.5
41	84.6	84.3	84.1	86.2	85.9	86.4	84.1	84.8	89.6	83.6	853.6
42	82.7	85.1	81.9	85.5	85.9	86.9	89.1	84.2	88.6	81.6	851.5
43	83.3	85.8	83.8	82.5	85.4	85.5	81.4	79.8	82.8	83.4	833.7
44	84.3	85.0	84.2	85.5	83.1	82.3	84.2	84.6	89.0	87.9	850.1
45	84.9	85.1	85.4	86.4	84.2	87.2	84.2	87.8	84.4	89.7	859.3
46	85.6	85.2	85.6	84.1	82.4	85.3	86.8	85.3	87.1	85.2	852.6
47	81.0	88.8	81.2	84.7	85.1	82.0	81.2	85.1	86.2	87.2	842.5
48	88.2	85.4	86.6	87.1	87.6	86.6	85.7	85.6	85.6	85.3	863.7
49	82.0	85.7	88.6	86.0	84.8	85.0	85.7	88.4	85.6	87.9	859.7
50	86.0	84.4	83.9	86.2	85.7	84.6	86.9	86.7	87.3	80.9	861.6
51	88.4	84.6	85.7	81.4	85.7	88.1	86.0	86.2	86.2	84.7	857.0
52	88.8	85.2	85.6	85.8	85.8	86.6	86.3	86.2	85.9	89.9	864.1
53	84.6	83.9	88.8	86.7	85.1	84.6	87.4	86.7	86.8	89.7	864.3
54	85.1	86.0	84.8	85.8	85.2	84.2	86.7	85.0	85.9	88.1	856.8
55	86.6	86.3	85.7	83.7	87.0	87.6	84.8	86.3	87.7	81.8	860.5
56	86.8	89.1	84.8	86.5	87.8	84.3	86.4	84.9	87.2	88.3	866.1
57	82.5	87.2	88.1	83.8	90.9	85.9	85.1	84.9	84.3	90.0	866.7
58	80.3	87.7	87.9	84.2	85.9	84.1	86.3	88.1	84.4	86.8	855.7
59	85.2	84.5	86.6	89.3	81.8	86.6	83.9	86.8	87.1	88.8	860.6
60	89.0	85.5	83.0	83.7	84.4	87.5	86.6	88.0	84.5	88.8	861.9
Total	5,095.9	5,072.6	5,086.7	5,079.4	5,074.9	5,085.9	5,082.4	5,090.8	5,169.8	5,150.5	50,988.0

TABLE 11 — Analysis of variance of apparent purity from 4-row plots 2 rods long, of which only the central rows were harvested

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	<i>z</i>
Between blocks	29	302.3527	10.4280	3.2289	0.3941
Within blocks	120	568.7860	4.7399	2.1771	
Total	149	871.1387	5.8466	2.4180	

## REGRESSION OF SUGAR PERCENTAGE ON YIELD AND OF APPARENT PURITY ON YIELD AND ON SUGAR PERCENTAGE

Calculations were made for the linear regression coefficients, within plots, of sugar percentage on yield and of apparent purity on yield and on sugar percentage for 4-row plots 2 rods long with only the central rows harvested. The regression coefficient expresses the expected value of the dependent variable on the basis of its relationship to the independent. These may be summarized as follows:

Regression	Regression coefficient (per cent)
Sugar percentage on weight.....	-0 020753
Apparent purity on weight.....	- 067567
Apparent purity on sugar percentage.....	3 334299

The significance of the regression coefficients was tested by the method proposed by Fisher<sup>14</sup>. The regression of sugar percentage on weight of beets was probably significant. The  $z$  test showed that the difference between the variations due to linear regression and to departure from regression exceeded the 5 per cent point but not the 1 per cent point. It may be concluded, therefore, that weight probably affected sugar percentage significantly when the relationship was determined within plots. The negative regression of purity on weight was not significant. The observed  $z$  value did not exceed the 5 per cent point in the latter case. The regression of apparent purity on sugar percentage was highly significant.

The regression equation may be used to express the estimated value of the dependent variable in relation to the independent variable. This is given in the case of regression of sugar percentage on weight by

$$Z = \bar{z} + b(w - \bar{w}),$$

where  $Z$  (Zucker) is the estimated sugar percentage and  $\bar{z}$  and  $\bar{w}$  are the means of sugar percentage and weight, respectively,  $w$  is any observed weight, and  $b$  is the regression coefficient. Letting  $P$  represent apparent purity, the different regression equations may be expressed as follows:

Regression	Regression equation
Sugar percentage on weight.....	$Z = 15.5646 - 0.020753 w$
Apparent purity on weight.....	$P = 88.2734 - 0.067567 w$
Apparent purity on sugar percentage.....	$P = 36.4588 + 3.334299 z$

In these equations  $w$  and  $z$  represent any observed value of weight and sugar percentage, respectively, obtained in the experiment.

The intraplot correlation coefficients may be given also, for convenience. The significant coefficients are in *italic*. If the regression coefficients are significant, it follows that the correlation coefficients must be significant also.

Correlation	Coefficient of correlation
Sugar percentage and weight.....	-0 1746
Apparent purity and weight.....	- 1210
Apparent purity and sugar percentage.....	.7096

There was little relationship between weight of beets and either sugar percentage or apparent purity. Sugar percentage and apparent purity were highly correlated, as would be expected.

<sup>14</sup> FISHER, R. A. Op cit (Footnote 10)

The observed value of  $z$  (0 1507) exceeds the 5 per cent point (0 1417) but not the 1 per cent point (0 2002). The two variances may, therefore, be considered as probably significantly different.

As an approximation to the sampling variance for apparent purity in plots 2 rods long, we may use 3 5064. This is on the assumption that the variance actually obtained from two 10-beet samples taken from adjacent rows within the plots would be very nearly the variance of two 10-beet samples where each was taken from both rows in a random manner. This variance would then be a measure of the sampling error and could be reduced by increasing the size of sample. The difference between the variance between plots and that within plots ( $4\ 7399 - 3\ 5064 = 1\ 2335$ ) would measure the variance due to soil differences between plots. This latter variance could be reduced only by increased replication. Approximately  $3\ 5064 - 4\ 7399$ , or 74 per cent of the variance between plots, was due to sampling error. Both variance within plots and variance

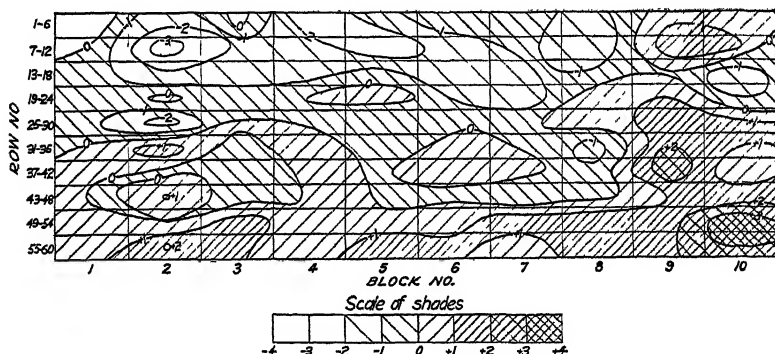


FIGURE 4.—Contour map of apparent purity of beets from one hundred 6-row plots, each 2 rods long, contour lines drawn through points deviating by  $-3$ ,  $-2$ ,  $-1$ ,  $0$ ,  $+1$ ,  $+2$ ,  $+3$  per cent from the mean apparent purity.

due to inherent soil differences between plots must be considered in estimating the value of replication and size of sample to reduce the standard error of the mean to a given level. Since the standard error for sugar percentage in percentage of the mean was greater than for apparent purity, it would follow that the standard error for purity would usually be lower than for sugar percentage with the same size of sample.

In Figure 4 is shown the same type of contour map for apparent purity as was given for weight and sugar percentage. These contour lines were drawn through the points at which the purity was 97, 98, 99, 100, 101, 102, and 103 per cent of the mean.

The contour lines bear a marked similarity to those for sugar percentage but not to those for yield. In general, the areas of high sugar percentage were also high in purity of juice and vice versa. Some exceptions are noted, however. Apparently the field was quite heterogeneous for weight of beets, sugar percentage, and apparent purity. While the two latter bear a distinct relationship to each other, there are marked differences in certain areas.

apparent purity and sugar percentage within plots. Correction on the basis of the regression of apparent purity on sugar percentage reduced the variance in apparent purity between plots from 5 8465 to 2 6669, or to 46 per cent of the original variance. Therefore 54 per cent of the variation in apparent purity between plots was due to the factors that affected sugar percentage.

These calculations substantiate the conclusion, arrived at previously, that the variation in weight was entirely independent of variation in purity and very nearly independent of variation in sugar percentage. Sugar percentage and apparent purity varied together to an appreciable degree. Slightly more than one-half of the variation in apparent purity was due to factors that affected sugar percentage as well.

### DISCUSSION

It would seem, from the data presented here, that fairly narrow plots, either 2 or 4 rods long, would be the most economical size and shape to use for agronomic experiments with sugar beets. Some modifications would need to be made for certain types of experiments. In regions where stands are known to be poor because of unfavorable soil conditions, diseases, or insect pests, larger plots would seem advisable or replication should be increased.

The standard errors obtained from using plots of varying size and shape probably could be considered fairly high estimates of the errors to be expected under average conditions. The contour lines for weight ran parallel to the direction of the rows to a very pronounced degree. This would result in an increased estimate of the standard error between plots. Under average conditions these contours probably would not parallel the direction of the rows to the same degree. The same was true of the sugar-percentage contours and, to a slightly less degree, of the apparent-purity contours. The estimates of the error between plots were probably slightly above that expected under average conditions, assuming environmental conditions similar to those of 1930.

The linear regression of sugar percentage on weight, for individual beet analyses, was found in a previous study<sup>15</sup> to be expressed by the equation

$$b = -0.589375 (w - \bar{w}),$$

where  $w$  was the weight of a single beet. A 1-pound increase in weight would then mean a reduction of 0.59 per cent sugar. In the present study the regression of sugar percentage on weight was expressed by the equation

$$b = -0.020753 (w - \bar{w}),$$

where  $w$  was the weight of a single-row plot 2 rods long. Each such plot contained a maximum of 33 beets. An average increase of 1 pound in weight per beet would mean a decrease of 0.68 per cent sugar (33 times  $-0.020753$ ), which is in fairly close agreement with the value found for the individuals. The regression of sugar percentage on weight was not entirely linear in the case of the individual beet analyses.<sup>15</sup> The quadratic regression showed that a unit increase

<sup>15</sup> IMMER, F. R. Op cit (Footnote 5)

The linearity of regression of sugar percentage on weight, apparent purity on weight, and apparent purity on sugar percentage was tested. The regressions were found to be linear.

VARIATION IN SUGAR PERCENTAGE AND IN APPARENT PURITY WHEN THEIR RELATIONSHIP WITH WEIGHT AND SUGAR PERCENTAGE, RESPECTIVELY, IS HELD CONSTANT

It would seem of interest to determine the variability in sugar percentage between plots after correction for regression of sugar percentage or weight within plots. The sum of squares of sugar percentage between plots after such correction would be given by

$$S\{(z-\bar{z})^2 - 2b(w-\bar{w})(z-\bar{z}) + b^2(w-\bar{w})^2\},$$

where  $S$  represents summation,  $(z-\bar{z})$  and  $(w-\bar{w})$  represent, respectively, any observed deviation of sugar percentage and weight between plots from their mean, and  $b$  is the regression coefficient of sugar percentage on weight within plots. Comparing this quantity with the departures of sugar percentage within plots from regression would give an exact test of the significance of variation in sugar percentage after correcting for its relationship with weight. The analysis of variance is shown in Table 14.

TABLE 14—*Test of variability of sugar percentage between plots apart from its relationship with weight*

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	$z$
Between plots, corrected for weight.....	149	73 9172	0 4961	0 7043	} 0 5817
Departure from regression.....	149	23 0954	1 550	3937	

The observed value of  $z$  exceeds the 1 per cent point, and it is concluded that the sugar percentage varied significantly apart from its relationship with weight. In fact, the variance between plots after correction for weight (0 4961) was 9 5 per cent greater than the variance without correction (0 4529). An explanation for this is found in the fact that the regression of sugar percentage on weight within plots was negative (−0 020753), while the sum of products,  $S\{(w-\bar{w})(z-\bar{z})\}$ , between plots was positive (95 9203).

The variation in apparent purity apart from its relationship with sugar percentage should prove of interest, since these two characters are highly correlated. The analysis of variance is shown in Table 15.

TABLE 15—*Test of variability of apparent purity between plots apart from its relationship with sugar percentage*

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	$z$
Between plots corrected for sugar percentage.....	149	397 3653	2 6869	1 6330	} 0 2100
Departure from regression.....	149	261 1234	1 7525	1 3238	

The observed  $z$  exceeds the 1 per cent point, indicating that apparent purity varied from plot to plot apart from the relationship between

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## METHODS OF DETERMINING BOUND WATER IN PLANT TISSUE<sup>1</sup>

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### INTRODUCTION

As a part of the research on the physiology of corn which was started when the European corn borer invaded Ohio, a study was made of the water content of different varieties of corn. This study included an attempt to find out the different ways in which water was held by the corn plant. In these studies, measurements were made of the amount of water removed from the tissues by pressure and the amount of bound water in the expressed sap. These measurements were suggested by the work of Newton and Gortner (17)<sup>3</sup> and Newton (16) on winter hardiness in wheat.

Unsatisfactory results were obtained in the measurements of the bound-water content of expressed sap from corn tissue by the cryoscopic method used by Newton and Gortner (17). Some determinations indicated no bound water in the sap, others showed a small percentage of bound water but satisfactory checks could not be obtained. These results were contrary to expectation, since the sap from leaf tissue was found to contain 4 to 5 per cent of colloidal material, largely proteins.

Before continuing work on the water content of corn, it was necessary, therefore, to test this method further, to try out other methods that had been suggested, and possibly to devise new ones; in other words, to make a critical study of methods used in determining bound water. Only those methods that appeared to be best suited for measuring bound water in plant tissues and in plant saps were considered.

### DEFINITION OF BOUND WATER

A satisfactory definition of bound water can hardly be made. All water that is not free water, that is, that does not show some of the common properties of liquid water, may be considered as bound water. Foote and Saxton (5, 6) recognize free, capillary or adsorbed, and combined water in such substances as lampblack, silica, alumina, and ferric oxide when mixed with water. They base their conclusions on the results of dilatometer measurements of the amount of water that will change to ice. Bouyoucos (1), also using dilatometer measurements, classified the water in certain soils as free and unfree. He states that unfree water may be either capillary adsorbed or combined.

<sup>1</sup> Received for publication Oct. 29, 1931, issued May, 1932. Investigations conducted in cooperation between the Division of Cereal Crops and Diseases, U. S. Department of Agriculture, and the Ohio Agricultural Experiment Station.

<sup>2</sup> The writer takes pleasure in acknowledging the helpful suggestions of F. D. Richey, of the Division of Cereal Crops and Diseases, during the preparation of this paper.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 686.

in weight did not result in as great a reduction in sugar percentage among the smaller beets as among the larger. One would expect, therefore, that when the sugar percentage was determined from bulk samples of entire beets the larger beets would contribute a greater quantity of juice of lower sugar content and a higher regression coefficient would be obtained. Such was actually the case.

The variability in sugar percentage between plots could not be reduced by means of the regression of sugar percentage on weight. Such regression was negative within plots and positive between plots. The variability of apparent purity between plots could be reduced 54 per cent by holding constant the effect of sugar percentage on purity. It would seem, therefore, that differences in apparent purity between varieties or treatments, apart from the effect of sugar percentage, could be determined with a high degree of accuracy. The general method of determining the variation in sugar percentage and apparent purity apart from their relationship with weight and sugar percentage, respectively, would seem to be extremely valuable in agronomic experiments with sugar beets.

#### SUMMARY

Studies of size and shape of plot in relation to field experiments with sugar beets have been made, and the relationship determined between weight, sugar percentage, and apparent purity.

Standard errors, expressed in percentage of the mean, decreased in general with increased size of plot. An explanation is offered to account for a greater standard error from 6-row plots than from 3 or 4-row plots, when the entire plot is harvested.

Efficiency in use of land decreased with increased size of plot when the entire plot was harvested. When the border rows of the plots were removed, 4-row plots were most efficient.

Weight of beets was significantly correlated (negatively) with sugar percentage, but not with apparent purity. Sugar percentage was highly correlated (positively) with apparent purity. Intraplot regression and correlation coefficients were given.

Contour maps for weight of roots, sugar percentage, and apparent purity were drawn from data on one hundred 6-row plots 2 rods long.

Sugar percentage varied significantly from plot to plot apart from its relation to weight. Fifty-four per cent of the variability in apparent purity between plots was due to factors that affected sugar percentage as well.

The sampling error was calculated for sugar-percentage and apparent-purity determination for 4-row plots 2 rods long. The manner in which the standard error between plots may be reduced by replication and size of sample has been demonstrated.

This paper reports the results of an attempt to simplify and standardize several of those that appear to be best suited for measuring bound water in plant tissues and in plant saps, as a preliminary to the determination of bound water in corn tissues.

#### METHODS OF MEASURING BOUND WATER

In most of the methods so far used, total water and free water are measured and the bound water is found by difference, the bound water being the water held by the solid part of the material in one or more of the several different ways discussed above

##### THE CRYOSCOPIC METHOD

The cryoscopic method was introduced by Newton and Gortner (17). The theory involved is the assumption that bound water does not dissolve sucrose

##### APPARATUS AND TECHNIC

A freezing-point apparatus, a refractometer, and some means of obtaining suitable samples of expressed sap are necessary in making determinations on plant material by this method. The writer modified the procedure followed by Newton and Gortner (17) and thereby shortened the laboratory manipulation but increased the amount of calculating necessary. In this modified method accurate weighings of the liquid and of the sucrose are both eliminated. The amount of sucrose added to the liquid, on which the excess depression of the freezing point depends, is determined with the refractometer used to obtain the total solid content of the material, as described by Gortner and Hoffman (8).

The modified procedure substitutes one reading with the refractometer for two analytical weighings. The method is as follows: Determine the refractive index and the depression of the freezing point of the original liquid, add sucrose to a similar portion, about 3 g to 10 ml; and after the sucrose is dissolved determine the refractive index and freezing-point depression of this mixture. Several readings with the refractometer are made on each sample and the results averaged, and duplicate freezing-point measurements are made on separate portions of each mixture. The refractometer readings are corrected to a temperature of 20° C and also for any difference between actual total solid content determined by vacuum oven drying and that indicated by the reading with the refractometer. From these data the amount of bound water is calculated by a formula modified slightly from the one given by Newton and Gortner (17).

##### DETERMINING AMOUNT OF SUCROSE ADDED

The refractometer was first used by Gortner and Hoffman (8) to determine the total solids in plant saps. This determination is based on the assumption that total solids in the sap have a refractive index very similar to that of sucrose. This assumption is true of rather dilute sols or solutions of dextrin, starch paste, glucose, fructose, gum arabic, and similar compounds, and of many plant saps. Before many determinations on any one substance are made, however, checks should be provided by vacuum oven drying. In some plant saps refractometer determinations are too high. Sap from blade tissue of corn gives values from 6 to 14 per cent too high, depending on the

Newton and Gortner (17) divide the water in plant saps into free water and bound water. Their method of determination is based upon the assumption that bound water will not dissolve sucrose.

In plant tissues and in plant saps where there is material in true solution, such as sugars and salts, water may be held by hydration of molecules and ions and by osmotic phenomena. Maximow (12) and Rosa (21) discuss these forms of water. Gortner (7) considers water of imbibition and water that will not dissolve sucrose as bound water. Meyer (13) considers water that can not be removed by pressure from the tissue after certain treatments as bound water. From these examples and from many others that might be cited it seems that no single standard has been established and that water may be considered as bound when it is held or contained in the material in any one of several different ways.

#### EVIDENCE FOR EXISTENCE OF BOUND WATER

There is considerable evidence in physical chemistry for the existence of bound water. The hydration of sucrose or the association of a certain number of water molecules with each sugar molecule is an example. Philip (19) states that 5 molecules of water are associated with each molecule of sugar, and Findlay (4) and Scatchard (22) consider 6 as the correct number. The hydration of sucrose can be proved by several separate and distinct methods by which actual measurements can be made. All methods indicate that the number of associated water molecules is either 5 or 6. Ions also may be hydrated. Water held by ions or molecules does not have some of the properties ordinarily shown by liquid water, such as vapor pressure, osmotic phenomena, etc., and may be considered as bound water.

Many substances in a state of fine subdivision, such as carbon black, silica gel, platinum black, and alumina, have the property of adsorbing substances, including water. The adsorbed water may be in a thin layer around the particles or in the minute spaces between the particles. It is held by very strong forces and may have properties quite different from those of liquid water. Those substances in plant tissues and in plant saps which are in a similar state of subdivision may also adsorb water. Adsorbed water may be considered as bound water.

In sols like gum arabic, which do not set to a rigid gel, some of the water is associated with the particles, probably similarly to water of hydration or adsorbed water. Water so held and also that in rigid gels like agar, gelatin, and silica gel and in other similar plant or animal products is considered by Gortner (7) as bound water. Water of imbibition held by substances like *Laminaria*, fibrin, starch grains, and mucilages is also considered by him as bound water. Changes in water content in these substances usually are associated with viscosity changes. The force required to remove water held by these substances is very great. Meyer (13) measured the water forced out of plant tissues by pressure after freezing at  $-15^{\circ}$  to  $-20^{\circ}$  C. and at  $-57^{\circ}$ . He considered that the water forced out without freezing corresponds roughly to the free water, and that that forced out after freezing, together with that left in the tissue, is bound in varying degrees.

Bound water does not exist in definite proportions relative to the solid material of the system, but as a ratio between bound water and free water. The ratio may be changed quickly by varying the temperature, acidity, surface energy, presence of electrolytes, pressure, etc. Various methods of measuring bound water have been suggested.

A comparison was made between this method of determining the amount of sucrose added to gum arabic solutions and the method of actually weighing the solution and the sucrose. The concentration of the gum arabic solution was determined with the refractometer and enough solution to contain 10 g of water was weighed out in a small weighing bottle. To this was added a weighed amount of sucrose. After the sucrose was dissolved the refractive index of the sugar-gum arabic mixture was determined. The amount of sucrose added was found from the two refractometer readings. The comparative data from a number of determinations which are given in Table 3 show that this method is essentially as accurate as weighing the sugar.

TABLE 3 — *Comparison of weighing and the refractive index method of obtaining the concentration of sucrose added to a gum arabic solution*

Gum arabic sol, percentage of total solids	Gum arabic	Gum arabic plus sucrose	Sucrose by difference	Actual amount of sucrose added
<i>Per cent</i>	<i>G per 1,000</i>	<i>G per 1,000</i>	<i>G per 1,000</i>	<i>G per 1,000</i>
9.6	103.5	449.4	342.9	342.2
9.6	103.5	449.4	342.9	342.2
9.6	103.5	448.4	341.9	342.2
9.6	103.5	449.5	342.1	342.2
9.6	103.5	452.0	344.6	342.2
9.6	103.5	449.0	341.6	342.2
Mean—	-----	-----	342.6	342.2
9.8	107.7	210.2	102.5	100.0
9.8	107.7	308.0	200.3	200.0
9.8	107.7	410.9	303.2	300.0

If the refractometer does not give the true measure of total solids in the material, the calculation of the results is somewhat more complicated. The following data, taken from determinations on sap from blade tissue of corn, illustrate how the calculations are made. Refractometer readings and the determinations of total solids by vacuum oven drying were made on the original sap, and refractometer readings on the sap after the sugar was dissolved. Exactly 3 g of sucrose was added to 10 ml of sap. The results further corroborate the fact that the amount of sucrose added can be found from refractometer readings on the sap.

COMPARISON OF REFRACTIVE INDEX METHOD AND WEIGHING FOR DETERMINING THE AMOUNT OF SUCROSE ADDED TO PLANT SAP

*Refractive Index Method*

Refractometer reading on original sap = 1.3604 at 20° C.

Percentage of total solids by refractometer = 17.7

Percentage of total solids by vacuum oven = 15.1.

Refractometer reading corresponding to 15.1 per cent total solids = 1.3559 at 20° C.

Correction to apply to refractometer reading  $1.3559 - 1.3604 = -0.0045$ .

Refractometer reading after adding sucrose = 1.3924 at 20° C (3 g to 10 ml of sap).

Corrected refractometer reading after adding sugar = 1.3879 at 20° C

Grams per 1,000 g solvent corresponding to  $\pi = 1.3879 = 510$

Grams per 1,000 g solvent corresponding to  $\pi = 1.3559 = 178$

Grams sucrose per 1,000 g solvent added =  $\frac{510 - 178}{1} = 332$

season of the year, while readings on sap from stem tissues of the same plants agreed almost exactly with the results of vacuum oven drying

The amount of sucrose added to a plant sap or to a gum arabic sol can be determined by the increase in refractive index, if the grams of total solids per 1,000 g of solvent are used instead of the percentages of total solids, that is, the grams of total solids per 1,000 g of solvent in the original sap subtracted from the grams of total solids per 1,000 g of solvent after sucrose was added will give the grams of sucrose per 1,000 g of solvent which was added. This method is the most convenient way of expressing the concentration of sucrose solutions when their freezing point is considered. From this value the additional depression of the freezing point due to the sucrose is determined and the percentage of bound water in the material calculated.

The refractive index, the percentage of total solids, and the grams of total solids per 1,000 g of solvent are given in Table 1. These data are based on the refractive indices of sucrose solutions given by Browne (2). The grams of total solids per 1,000 g of solvent were calculated by simple proportion from the percentages of sucrose and the percentages of water. Temperature corrections added to all values when readings are made above 20° C are given in Table 2. These data should be put in graphic form to be useful in determining the concentration of the solutions or mixtures.

TABLE 1.—Percentage by weight, refractive index, and grams of total solids per 1,000 g of solvent of sucrose solutions

Percentage by weight	Refractive index at 20° C *	Grams per 1,000 g solvent	Percentage by weight	Refractive index at 20° C	Grams per 1,000 g solvent	Percentage by weight	Refractive index at 20° C	Grams per 1,000 g solvent
1.....	1 3344	10 1	15.....	1 3557	176 5	28.....	1 3775	388 9
2.....	1 3359	20 4	16.....	1 3573	190 5	29.....	1 3793	408 5
3.....	1 3374	30 9	17.....	1 3590	204 8	30.....	1 3811	428 6
4.....	1 3388	41 7	18.....	1 3608	219 5	31.....	1 3829	449 3
5.....	1 3403	52 9	19.....	1 3622	234 6	32.....	1 3847	470 6
6.....	1 3418	63 8	20.....	1 3639	250 0	33.....	1 3865	492 5
7.....	1 3433	75 3	21.....	1 3655	265 8	34.....	1 3883	515 2
8.....	1 3448	87 0	22.....	1 3672	282 1	35.....	1 3902	538 5
9.....	1 3464	98 9	23.....	1 3689	298 7	36.....	1 3920	562 5
10.....	1 3479	111 1	24.....	1 3706	315 8	37.....	1 3939	587 3
11.....	1 3494	123 6	25.....	1 3723	333 3	38.....	1 3958	612 9
12.....	1 3510	136 4	26.....	1 3740	351 4	39.....	1 3978	630 3
13.....	1 3526	149 4	27.....	1 3758	369 9	40.....	1 3997	666 7
14.....	1 3541	162 8						

\* From Schonrock's table as given by Browne (2, p 64)

TABLE 2 — Temperature correction for total solids by the refractometer

[Add the value to the total solids to convert values to 20° C. Computed from Stanek's correction tables given by Browne (2)]

Temperature (°C)	Correction for indicated number of grams of total solids per 1,000 g of solvent							
	52 6	111 1	176 5	250 0	333 3	428 6	538 5	666 7
21.....	0 6	0 8	0 8	0 9	0 9	1 0	1 1	1 2
22.....	1 3	1 6	1 6	1 8	1 9	2 0	2 2	2 3
23.....	1 9	2 2	2 2	2 4	2 6	3 0	3 2	3 5
24.....	2 5	2 9	3 1	3 4	3 6	4 0	4 3	4 7
25.....	3 2	3 6	3 8	4 3	4 7	5 1	5 5	6 0
26.....	3 8	4 3	4 6	5 1	5 6	6 1	6 6	7 2
27.....	4 5	5 1	5 4	6 0	6 6	7 1	7 8	8 5
28.....	5 3	5 9	6 2	6 9	7 6	8 3	9 0	9 8
29.....	6 0	6 7	7 2	7 8	8 5	9 4	10 2	11 1
30.....	6 7	7 4	8 2	8 9	9 7	10 5	11 5	12 5

The corrections for undercooling in determining freezing points, taken from Harris (9), are included here (Table 5) so that complete data will be available for making determinations of bound water by the cryoscopic method

TABLE 5—Undercooling correction factor for freezing-point determinations

Tenth degrees	Whole degrees			Tenth degrees	Whole degrees		
	0	1	2		0	1	2
0 0	0 000	0 987	0 975	0 5	0 993	0 981	0 968
1	998	986	973	6	992	980	967
2	997	985	972	7	991	978	966
3	996	983	971	8	990	977	965
4	995	982	970	9	988	976	963

#### CALCULATING RESULTS

The percentage of bound water in the liquid or solution is calculated by a formula similar to the one given by Newton and Gortner (17)

$$\frac{\Delta_2 - (\Delta_1 + K_m)}{\Delta_2 - \Delta_1} \times C = \text{per cent bound water}$$

$\Delta_2$  is the depression of the freezing point after adding sucrose

$\Delta_1$  is the depression of the freezing point of the original solution

$K_m$  is obtained from the difference between the two concentrations, corresponding to the two refractive index readings (Table 1), and the depression of the freezing point of a sucrose solution (Table 4)

$C$  is the percentage of free water in a sucrose solution of the concentration used (Table 5)

Inasmuch as exactly 342.2 g of sucrose is not always used,  $K_m$  will not always be 2.085° C., but some other value, depending on the amount of sucrose added.  $C$  also will vary with the amount of sucrose added. These values can best be obtained from graphs of the values for various strengths of sucrose approximating molecular concentrations, given in Tables 1 and 4 and in the tabulation on p. 674

#### ADVANTAGES AND DISADVANTAGES OF THE METHOD

The advantage of the modified cryoscopic method is that the actual laboratory procedure is shortened, although the calculations of the results are more complicated. When only a few determinations are to be made, the original method is superior, but when many measurements are to be made, graphs of the various constants used in the determination can be used as an aid in calculation, and the shorter laboratory procedure is the more efficient. From six to eight different saps or solutions can be analyzed with duplicate freezing-point depression determinations in a half day with this short method.

The disadvantage of the cryoscopic method is that it can be used only with liquid or semiliquid material. This is particularly a disadvantage when plant tissues are being studied, as the amount of bound water in the tissue is the most important consideration. Furthermore, the method measures only water that is not free to dissolve sucrose. If water held by gelatin and agar is considered as

*Weighing method*

3 g sucrose to 10 ml original sap  
 Specific gravity of original sap = 1.070 at 20° C  
 3 g sucrose to 10.70 g sap  
 10.70 g sap contain  $(1.00 - 0.151) \times 10.70$ , or 9.084 g water  
 3 g sucrose to 9.084 g water, or 330 g sucrose to 1,000 g water  
 Grams sucrose per 1,000 g solvent added = 330

## DETERMINING DEPRESSION OF FREEZING POINT OF SUCROSE SOLUTIONS

The additional depression of the freezing point of the mixture, due to the added sucrose, is proportional to the amount of sucrose added. Newton and Gortner (17) used 2.085° C as the molecular depression for sucrose, assuming 6 molecules of water of hydration for each molecule of sucrose (Data of Scatchard (22)). A number of determinations were made to check this value, but the observed depressions were less than the calculated.

Table 4 gives the results of a series of these determinations. Each of the observed depressions is the average of four separate determinations on each concentration of the sucrose solution. The values calculated on the assumption that a molecular solution freezes at -2.085° C are given in one column. If both the observed and the calculated values are plotted, two parallel lines are formed, that for the observed depressions being about 0.037° C. lower than the other. This difference may be due to some systematic error in all the freezing-point measurements, such as the purity of the sucrose used, the degree of undercooling, or the calibration of the thermometer. The values are very consistent, however, and the same procedure was used in making all the freezing-point measurements. These values accordingly have been used in this work rather than those given by the other authors.

TABLE 4.—Comparison of actual determinations of the freezing point of sucrose solutions and calculated values of the same solution

Percent- age of sugar by weight	Sucrose per 1,000 g of water	Observed depression of freezing point	Calcu- lated depression of freezing point	Differ- ence
<i>Per cent</i>	<i>Grams</i>	<i>° C</i>	<i>° C</i>	<i>° C</i>
14	162.8	0.955	0.992	0.037
16	190.5	1.124	1.161	0.037
18	219.5	1.292	1.337	0.045
20	250.0	1.485	1.523	0.038
22	282.1	1.685	1.719	0.034
25.5	342.2	2.054	2.085	0.031

The percentages of free water in different concentrations of sucrose solutions are given in the following tabulation:

200 g of sucrose per 1,000 g solvent.....	94.5 per cent of free water
250 g of sucrose per 1,000 g solvent.....	93.1 per cent of free water
300 g of sucrose per 1,000 g solvent.....	91.7 per cent of free water
350 g of sucrose per 1,000 g solvent.....	90.3 per cent of free water
400 g of sucrose per 1,000 g solvent.....	90.0 per cent of free water.

These values were calculated from the molecular weights of water and of sucrose and the actual freezing points of the various sucrose solutions as given in Table 4.

## CALIBRATING THE CALORIMETER SYSTEM

Although the actual determinations are quite simple, the work necessary to calibrate the system and to calculate the results is considerable. The factor for the calorimeter system is obtained by putting ice into water in the calorimeter and determining how much heat is necessary to melt it and raise the temperature of the resulting water to the equilibrium temperature of the system. For a known weight of ice this amount of heat can be calculated from the latent heat of fusion of ice, 79.75 calories, and the specific heat of water. Any difference between the observed and the calculated values must be due to transfer of heat from the calorimeter system. The quotient of these two values gives a factor for converting the observed values to the true values for the material in the experiments.

A 25 g sample of the material has been used. By putting about 25 g of water in the freezing tubes and carrying out the determinations just as was done with tissue or other material, a factor for the calorimeter system was determined which included any error due to taking up heat while transferring the material from the freezing chamber. Table 6 gives the observed and calculated values of heat necessary to melt the ice and warm the water to the equilibrium of the system, and the corresponding factor for the system. This factor applies, of course, only to the system of thermos bottle, stirrer, thermometer, etc., used in these particular experiments.

TABLE 6—*Determination of the factor for the calorimeter system*

Ice used (grams)	Calories required to melt ice and warm water to equilibrium	Calories given up by 500 cc water cooling to equilibrium	Factor for the calorimeter system	Ice used (grams)	Calories required to melt ice and warm water to equilibrium	Calories given up by 500 cc water cooling to equilibrium	Factor for the calorimeter system
23.96.....	2,768.4	2,587.4	1.0700	24.05.....	2,774.8	2,602.4	1.0662
21.40.....	2,481.7	2,317.7	1.0708	22.59.....	2,613.5	2,442.6	1.0700
24.11.....	2,779.6	2,597.4	1.0701	24.22.....	2,793.3	2,617.4	1.0672
23.58.....	2,719.6	2,542.5	1.0697	24.10.....	2,778.9	2,607.4	1.0658
23.78.....	2,736.1	2,557.4	1.0699				
24.20.....	2,787.1	2,612.4	1.0669	Mean.....			1.0684
23.50.....	2,710.4	2,542.5	1.0660				

## CALCULATING RESULTS

The fall in temperature of the water in the calorimeter multiplied by the specific heat of water multiplied by the volume of water and the factor for the calorimeter system gives the total number of calories required to melt the ice and warm the water and dry matter of the tissue up to the equilibrium temperature. This heat is used in several ways: (1) To warm the dry matter of the tissue from its temperature when placed in the calorimeter to the equilibrium temperature. This amount can be determined from the specific heat of the material and the change in temperature. (2) To warm the water in the tissue from the freezing (thawing) temperature of the tissue to the equilibrium temperature. This amount can be determined from the amount of water, its specific heat, and the change in temperature. The approximate freezing point of similar tissue should be known. The determinations from (1) and (2) are added, and this sum is subtracted from

bound water, then this method fails to give a real measure of bound water, since much of the water held by those substances will dissolve sucrose. Gortner (7) has outlined certain other objections to the method. It probably gives minimum values for bound water. It presupposes, (1) that bound water is not changed to free water by the freezing process, (2) that the addition of sucrose does not alter the bound-free water ratio, (3) that none of the water that is firmly associated with the colloids is free to dissolve sucrose, (4) that there is no hydrolysis of the sucrose by acids or by enzymes in the material, and (5) that no adsorption of sucrose by the solid matter occurs. None of these assumptions is probably entirely correct.

#### THE CALORIMETER METHOD

The calorimeter method was first introduced by Muller-Thurgau (14, 15) and recently has been used by Thoenes (23) and Robinson (20). The method is based on the assumption that bound water does not freeze. Since free water changes to ice and it requires 79.75 calories to melt each gram of ice while unfrozen water below 0° C. has a specific heat of approximately 1.00, the amount of heat required to melt the mass of frozen material can be used to determine how much water was frozen.

#### APPARATUS AND TECHNIC

The equipment necessary for determinations by this method consists of a calorimeter thermometer, a calorimeter, freezing tubes for the tissue or material, low-temperature thermometers, and a freezing cabinet or refrigerator where a temperature as low as -25° C. or lower can be maintained. Quart-size all-steel thermos food jars make excellent calorimeters for plant-tissue work.

In making determinations the material is weighed and placed in freezing tubes. The tubes are about 4 inches long, open at both ends, and about one-half inch larger in diameter at one end than at the other. This kind of tube is necessary in order to make a quick transfer of the frozen material to the calorimeter without thawing any of it, as is likely to occur if the tubes are of uniform diameter. Both ends are closed tightly with rubber stoppers. The tubes of material are placed in a freezing room or cabinet at about -25° C. overnight. Before the measurements are made the tubes are taken in the hand and the frozen material is thawed around the tube and pushed to the large end, where it is not in contact with the wall of the tube. One tube has a thermometer in it. When the tubes have come to equilibrium again, as indicated by the thermometer, they are taken one at a time and the contents quickly transferred to the water in the calorimeter. The volume of water and its temperature to 0.01° C. are known. The tissue and water are stirred until equilibrium is reached and the fall in temperature of the mixture is determined.

Any loss of heat from the calorimeter during the stirring is reduced to a minimum by adjusting the temperature of the water in the calorimeter so that the fall in temperature will be about equal on either side of room temperature. This range can be found out by a preliminary determination on similar material. The material is stirred steadily until the fall in temperature in the bottle ceases. Finely minced or ground material comes to equilibrium much more quickly than large chunks. The time required to reach equilibrium is only a few minutes. Six to eight determinations can be made in an hour.

investigation, since this does not depend on the size of the sample or its water content  $SW(T_o + T_e) + sM(T_o + T_e)$  can also be shortened to  $SM(T_o + T_e)$ .  $S$  now is the mean specific heat of the sample and  $M$  is its green weight. The specific heat of the green sample can not be determined for the entire temperature range,  $T_o + T_e$ , since ice will form below the freezing point of the material. It can be determined with sufficient accuracy for most work by using the temperature range  $0^\circ\text{C}$  to  $T_e$  and assuming that it does not change materially below  $0^\circ\text{C}$ , or it can be calculated from the specific heat of the dry matter of the sample.

Thoenes (23) and Robinson (20) have simplified the formula still further by assuming that the specific heat of ice is 0.500 and of water below zero 1.000. The formula that they use is given by Gortner (7)

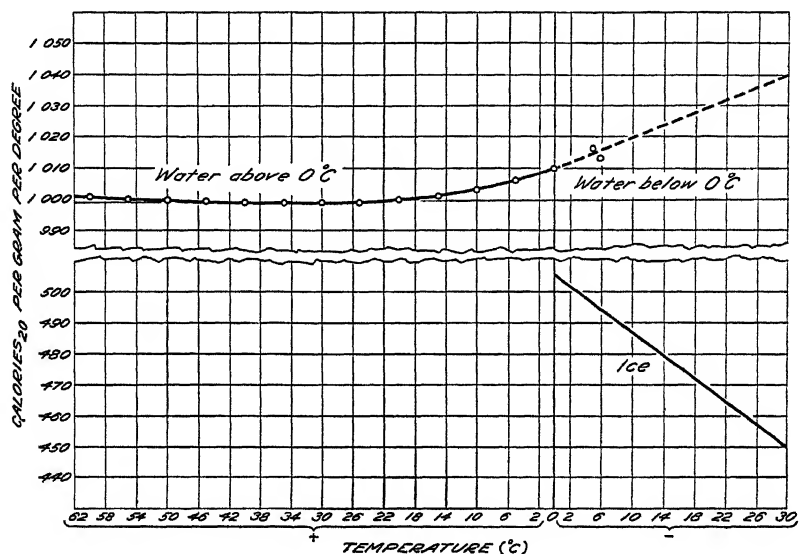


FIGURE 1.—Specific heat of water and of ice

Measurements by the Bureau of Standards (3) of the specific heat of ice, however, show that the specific heat of ice is decreasingly lower than 0.500 at temperatures much below  $0^\circ\text{C}$ . The specific heat of water below zero, on the other hand, increases as the temperature is lowered.

Table 7 gives the specific heat of water both above and below  $0^\circ\text{C}$ . and the specific heat of ice. The values for ice were taken from Dickinson and Osborne (3). The values for water above zero were taken from the Handbook of Chemistry and Physics (10). They were reduced to values at  $20^\circ\text{C}$ , to be comparable with ice. The values for water below zero were obtained by extending the curve of values from  $0^\circ$  to  $-30^\circ$  as a straight line, assuming that the specific heat of water continues as a linear function of the temperature, as shown in Figure 1, no extensive data being obtainable.

the total number of calories required. The remainder (3) is the number of calories required (a) to warm the unfrozen water from its temperature when placed in the calorimeter to the freezing (thawing) point of the tissue, (b) to warm the ice from its temperature when placed in the calorimeter to the freezing (thawing) point of the tissue, and (c) to melt the ice.

The total water in the sample, the latent heat of fusion of ice (79.75 calories), and the specific heat of water and of ice are known. The amount of free or frozen water accordingly can be calculated by simultaneous equations. Letting  $X$ =free or frozen water,  $Y$ =bound or unfrozen water, and  $W$ =total water in the sample, then

$$X + Y = W \quad (1)$$

Let  $C$  be the number of calories required to warm the ice and the unfrozen or bound water to the melting (thawing) point and to melt the ice.  $T_o$  is the temperature of the material at the time it was placed in the calorimeter, and  $T_\Delta$  is the freezing point of similar material. Let  $SI$  be the mean specific heat of ice, and let  $SBW$  be the mean specific heat of unfrozen or bound water. Then

$$C = X [79.75 + SI (T_o - T_\Delta)] + Y [SBW (T_o - T_\Delta)] \quad (2)$$

Combining equations (1) and (2) and equating to  $X$ ,

$$X = \frac{\frac{C}{SBW (T_o - T_\Delta)} - W}{\frac{79.75 + SI (T_o - T_\Delta)}{SBW (T_o - T_\Delta)} - 1}$$

or simplifying,

$$X = \frac{C - W [SBW (T_o - T_\Delta)]}{79.75 - [T_o - T_\Delta] (SBW - SI)} \quad (3)$$

Now,

$$C = FNS (T - T_e) - [SW (T_\Delta + T_e) + sM (T_o + T_e)] \quad (4)$$

where  $F$  is the factor for the calorimeter system,  $N$  is the amount of water used in it (500 ml in this work),  $S$  is the mean specific heat of water for the temperature range indicated,  $T$  is the temperature of the water in the calorimeter before the sample was placed in it,  $T_e$  is the temperature in the calorimeter at equilibrium after the sample was introduced,  $s$  is the mean specific heat of the dry matter of the sample for the temperature range indicated,  $M$  is the amount of dry matter in the sample.

By combining equations (3) and (4) the complete formula becomes

$$X = \frac{FNS (T - T_e) - [SW (T_\Delta + T_e) + sM (T_o + T_e)]}{79.75 - [T_o - T_\Delta] (SBW - SI)}$$

Bound water =  $W - X$ .

The individual calculations can be shortened somewhat by solving the denominator of the fraction for all values of  $T_o - T_\Delta$  used in the

## APPARATUS AND TECHNIC

The determinations are carried out in flasks with long, narrow, graduated necks, called dilatometers. The dilatometers are immersed in freezing mixtures of any desired temperature. The material is covered with petroleum ether, which is immiscible with water. The expansion is obtained as freezing or cooling occurs by noting the change in the meniscus of the petroleum ether in the narrow neck.

No regular dilatometers were available, and cream-test bottles were used instead. They are suitable for most substances, but filling them with solid material is tedious. The material must be in small pieces to go through the narrow neck of the flask. They have one advantage over the form of dilatometer used by Bouyoucos (1), however, in that they are not hard to seal, having only one opening. The neck is graduated to 0.05 ml, and readings can be estimated to 0.01 ml. A 25+g sample of liquid material can be used in them very well.

The sample is introduced into the bottle, covered with petroleum ether, and the whole cooled in a freezing mixture to any desired temperature. The expansion due to ice formation gives the amount of water that freezes at this temperature. Bouyoucos (1) supercooled his soil samples to  $-1.5^{\circ}$  and  $-4^{\circ}$  C and took the volume of the expansion at these temperatures. Rosa (21) made determinations at  $-3^{\circ}$ ,  $-4^{\circ}$ ,  $-5^{\circ}$ , and  $-6^{\circ}$ . Foote and Saxton (5, 6) made readings on their dilatometers at a series of temperatures ranging from  $0^{\circ}$  to  $-30^{\circ}$ .

Bouyoucos (1) considered the water in soils that froze at  $-1.5^{\circ}$  C as free water, and that which froze at  $-4^{\circ}$  as capillary adsorbed water, and that unfrozen at  $-4^{\circ}$  as combined water. Foote and Saxton (5, 6) could find no temperature where the different forms of water could be sharply separated. They expressed their results as curves and show that the amount of water that freezes increases as the temperature is lowered in some cases, while in others it is all frozen at a quite definite point. They further show that, after the water in some substances is frozen, the temperature may be raised to a point above which freezing occurred when cooling the material, without melting any of the ice. This means that ice may be superheated.

## PROCEDURE

The general procedure used is similar to that of other workers who have employed the dilatometer method, except that the order of taking the readings on the dilatometer was reversed. The material was placed in the dilatometer and frozen solid overnight, and the petroleum ether was then added. A series of readings on the volume of the material in the dilatometer as the temperature was changed was made, after which the material was allowed to melt and another series of readings was made on the volume as the temperature was changed. This procedure was necessary to prevent breaking the dilatometers, which were placed in a temperature cabinet and could not be shaken or stirred as freezing occurred. When the ether was added before freezing, ice formed first between the ether and the material, preventing expansion as freezing progressed, and the bottles were usually broken.

TABLE 7—*Specific heat of water and of ice*  
[Calories<sub>20</sub> per gram per degree]

Temperature (° C)	Water below zero	Ice	Water above zero	Temperature (° C)	Water below zero	Ice	Water above zero
0.....	1 010	0 506	1 010	16.....	1 026	0 176	1 001
1.....	1 011	504	1 009	17.....	1 027	174	1 001
2.....	1 012	502	1 008	18.....	1 028	472	1 000
3.....	1 013	500	1 008	19.....	1 029	470	1 000
4.....	1 014	498	1 007	20.....	1 030	468	1 000
5.....	1 015	496	1 006	21.....	1 031	467	1 000
6.....	1 016	495	1 005	22.....	1 032	465	1 000
7.....	1 017	493	1 005	23.....	1 033	463	999
8.....	1 018	491	1 004	24.....	1 034	461	999
9.....	1 019	489	1 004	25.....	1 035	459	999
10.....	1 020	487	1 003	26.....	1 036	457	999
11.....	1 021	485	1 003	27.....	1 037	455	999
12.....	1 022	483	1 002	28.....	1 038	454	999
13.....	1 023	481	1 002	29.....	1 039	452	999
14.....	1 024	480	1 002	30.....	1 040	450	999
15.....	1 025	478	1 001				

#### ADVANTAGES AND DISADVANTAGES OF THE METHOD

The calorimeter method of determining bound water has been very satisfactory. It has certain advantages in ease of technic, and it can be used for any kind of material, whether liquid, semiliquid, or solid. For these reasons it appears to be the best method so far suggested for measuring bound water in plant tissues. It measures water that does not freeze at the temperature to which it is exposed.

The calorimeter method is open to only one of the several objections that were listed under the disadvantages of the cryoscopic method, namely, the assumption that the bound water-free water ratio may not shift during the freezing process. In substances with no material in true solution most of the water is frozen at a few degrees below zero and a lower temperature does not crystallize any more of it. But where there is considerable material in true solution, as in plant tissues, more ice may separate as the temperature is lowered. For this reason all reported measurements of bound water in plant tissues or plant saps by this method should include a statement of the temperature at which the material was frozen, and comparisons should be made only between materials frozen at the same temperature.

#### THE DILATOMETER METHOD

The dilatometer method has been used to determine the amount of water that will freeze in certain systems at certain temperatures. Foote and Saxton (5, 6) used this method in determining water held in lampblack, silica, ferric oxide, and alumina. Bouyoucos (1) determined free and unfree water in soils by this method. McCool and Miller (11) and Rosa (21) used it in determining the amount of water in plant tissues which would not freeze at certain temperatures. The principle involved in the method is the expansion that occurs when water changes to ice. This expansion is about one-tenth the volume of the water that changes to ice, and from it the amount of water that freezes can be determined. Bound water is found by difference from total water.

## COMPARISON OF THE THREE METHODS OF MEASURING BOUND WATER

In order to compare these three methods, measurements of bound water by each method were made on exactly similar material. Gum arabic was chosen as material because it was the substance originally used by Newton and Gortner (17) and because a large supply could readily be obtained. All measurements in this comparison were made on the same lot of gum arabic, which was ground and thoroughly mixed to insure a uniform sample. It was originally intended to check all methods on several different materials, but this was found impossible because of lack of time. The results recently published by

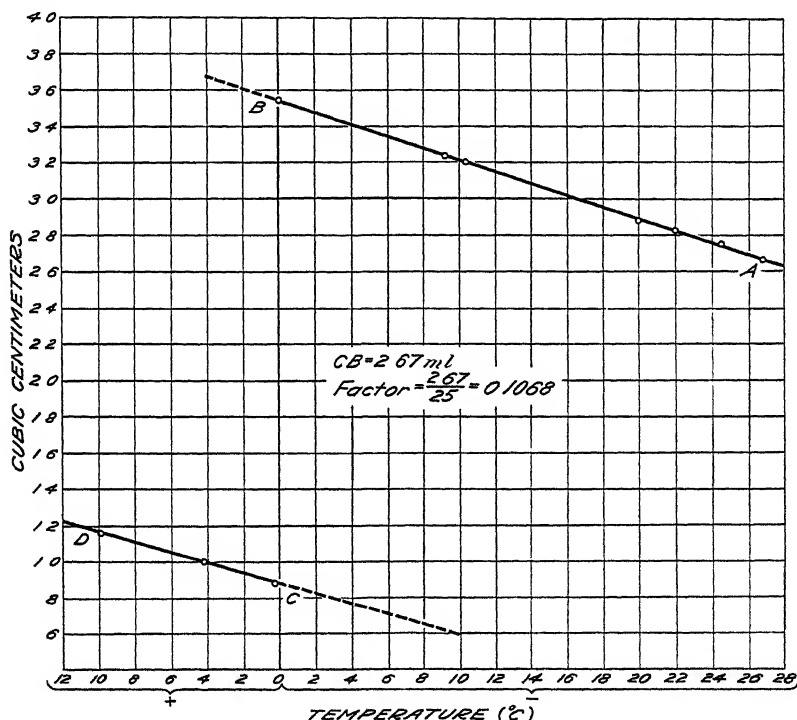


FIGURE 2—Calibration of dilatometer No 3 A-B=change in volume of 25 g of ice, petroleum ether, and the dilatometer with changes in temperature, C-D=change in volume of 25 ml of water, petroleum ether, and the dilatometer with changes in temperature, B-C=expansion due to ice formation

Newton and Martin (18) on the bound-water content of different substances by the cryoscopic method would suggest that some very interesting results might be obtained if the materials that they used were checked by several methods.

Experience soon showed that all measurements would have to be made as soon as possible after the solution was prepared and all on the same solution, as changes occur as the solutions stand and two solutions apparently made up in the same way may not have the same bound-water content. After a considerable number of preliminary experiments, a saturated solution (18.6 per cent) of gum arabic was prepared, and the values for bound water in Table 8 were all obtained on this solution.

## CALIBRATING THE DILATOMETER

Each dilatometer must be calibrated separately, since the glass in each is of different thickness and all do not have the same volume. The graduations on the neck are standard and uniform. The factor obtained represents the expansion due to ice formation and any other changes in volume of the glass vessel due to temperature changes, and thus the values are usually higher than if they were due to ice formation alone. Since the factor obtained from the calibration may vary with the rate and temperature at which the ice formed, it is essential that they be used at the same temperature, etc., as those for which they are calibrated.

In calibrating the dilatometers, 25 ml of water was placed in the dilatometer and frozen solid overnight in the freezing cabinet at  $-25^{\circ}\text{C}$ . The dilatometers were filled with petroleum ether up to the top graduation on the neck and corked. After temperature equilibrium was reached the position of the meniscus in the graduated neck was recorded. The temperature of the cabinet was changed by about  $5^{\circ}$  intervals, and after temperature equilibrium had been reached, in two or three hours, the meniscus was again read. A series of such readings was obtained at intervals until the material was at a temperature at which it melted. After all the material had melted another series of readings was made at  $0^{\circ}$  and at intervals of about  $5^{\circ}$  up to room temperature. When the data were plotted two lines were obtained. One of these represented the change in volume of the unfrozen material and the other the change in volume of the frozen material, with changes in temperature. The distance between the two lines, which are usually straight and parallel, is the expansion in milliliters due to ice formation of the 25 ml of water. A graphic solution of the factor for one of the dilatometers is given in Figure 2.

In the determination of bound water in samples of various materials the procedure was the same as in determining the factor with water for the dilatometers. The change in volume then was corrected for the dilatometer used in accordance with the factor for it which had been determined. Thus it is essential that all determinations on bound water in different samples be carried out under the same conditions under which the dilatometers were calibrated.

## ADVANTAGES AND DISADVANTAGES OF THE METHOD

This method can be used to measure bound water in almost any kind of material, either liquid or solid. With the cream-test bottles used as dilatometers, the method is practically restricted to liquid or semiliquid material, because of the small neck of the flask. The method is simple and accurate and involves no complicated calculation as do the other two methods, since results can be obtained by graphic solution. It is free from errors when properly used. The only precautions necessary are to see that the dilatometers are tightly stoppered to prevent evaporation of the petroleum ether, and to be certain that all the air is removed from the material before the readings are made. This latter is very difficult with plant tissue, especially mesophyll tissue containing intercellular spaces. One decided advantage of the method is that the amount of water that changes to ice at each temperature can be obtained. It measures water that will not freeze at different temperatures.

by that method. Thus, a series of determinations of bound water in samples of corn tissue had a standard deviation of only 0.81, or some 4.2 per cent of the indicated bound-water content.

An attempt was made to check the three methods on expressed sap from corn leaf tissue. The percentage of bound water by the calorimeter method was 14.8 per cent, by the dilatometer method 12.7 per cent, and by the cryoscopic method 5.8 per cent. All three methods were used on the same sample of expressed sap. The results by the calorimeter method and by the dilatometer method are in reasonably good agreement, but the results by the cryoscopic method are not. The cryoscopic method was very good for determining bound water in gum arabic solution. It has also been used with apparent success by other workers, Meyer (13) and Newton and Martin (18), on many different plant saps and other materials. However, as previously stated, it failed to give reliable results with expressed sap from corn tissue.

Since the cryoscopic method does not always give reliable results, determinations of bound water should be checked by different methods whenever possible.

If all three methods were equally accurate, the calorimeter method would apparently be the more useful, since it is easy to use with any type of material. The bound-water contents of a number of different substances determined by the calorimeter method are given in Table 9 as an illustration.

TABLE 9—Total water, free water, and bound water content of different materials, determined by the calorimeter method

Material	Date	Total water	Free water	Bound water			Temperature at which material was frozen	Temperature at which material was determined	Treatment of tissue
				Grams	Per cent	Grams			
	1930	Per cent	Grams	Grams	Per cent	Grams	° C	° C	
Buckeye twigs.....	Feb 28	56.0	26.2	29.8	53.2	67.8	-23.0	-4.5	Shavings
Buckeye buds.....	do	54.0	24.5	29.5	54.6	64.2	-23.0	-4.0	Whole
Maple twigs.....	Mar 4	44.0	20.4	23.6	53.6	42.2	-24.0	-5.5	Shavings
Pine needles.....	Mar 6	56.0	40.7	15.3	27.3	34.8	-23.0	-11.5	Ground
Do.....	do	56.0	41.2	14.8	26.4	33.7	-23.0	-11.0	Minced
Do.....	do	42.0	23.1	18.9	45.0	32.6	-23.0	-11.0	Press cake
Do.....	Apr 1	56.8	40.8	16.0	28.2	37.0	-22.0	-20.0	Minced
Do.....	do	56.8	38.9	19.9	35.0	46.1	-12.0	-10.0	Do
Carbon.....	Mar 27	60.0	28.2	21.2	42.4	42.4	-23.0	-3.0	
Filter paper.....	do	73.0	61.6	11.4	15.6	42.5	-23.0	-4.2	
Starch paste.....	do	98.5	94.4	4.1	4.2	27.3	-23.0	-2.5	
Corn blade.....	Aug 12	72.2	56.6	15.6	21.6	56.1	-25.0	-25.0	Do
Do.....	do	72.2	56.8	15.4	21.3	55.4	-25.0	-25.0	Ground
Corn sheath.....	do	73.3	67.9	16.4	13.3	47.9	-25.0	-25.0	Do
Corn stem (upper).....	do	78.3	69.5	8.8	11.2	40.5	-25.0	-25.0	Do
Corn stem (middle).....	do	80.0	73.8	6.2	7.8	31.0	-25.0	-25.0	Do
Corn stem (lower).....	do	80.9	74.5	6.4	7.9	33.5	-25.0	-25.0	Do

To all three methods the objection may be made that they measure bound water at or near the freezing point. Since bound water is in equilibrium with the free water of the system, and this equilibrium may be changed by temperature, they do not give a true measure of the bound water present at ordinary temperatures. This objection is not serious when bound water is considered in relation to cold

TABLE 8—Comparison of the three methods of measuring bound water in an 18.6 per cent gum arabic solution

## CRYOSCOPIC METHOD

Sucrose added	$K_m$	$C$	$\Delta_2$	Bound water
	$^{\circ}C$	Per cent		Per cent
260 8.....	1 552	92.9	1 827	10.84
403 9.....	2 431	88.9	3 018	15.59
305 5.....	1 830	91.6	2 182	12.23
267 2.....	1 592	92.6	1 922	13.00
289 0.....	1 728	92.0	2 035	11.10
309 2.....	1 852	91.5	2 173	10.92

 $\Delta_1 = 0.070^{\circ}C$ 

Mean = 12.28

 $\sigma = 1.83$ PE<sub>m</sub> =  $\pm 0.50$ 

## CALORIMETER METHOD

$T_o$	$T$	$T_s$	$FNS(T-T_s)$	$SW(T_o-T_s)$	$sM(T_o-T_s)$	$\frac{79.75-T_o-T_s}{T_{\Delta}(SBW-SI)}$	Bound water
$^{\circ}C$	$^{\circ}C$	$^{\circ}C$	Calories	Calories	Calories	Calories	Per cent
-4.0	27.32	23.36	2113.32	578.87	74.33	77.7610	10.59
-3.8	27.92	23.98	2102.64	587.76	75.47	77.8630	11.97
-3.5	27.24	23.40	2049.28	569.14	73.08	78.0194	14.12
-3.4	27.54	23.60	2102.64	571.25	73.35	78.0703	11.07
-3.3	27.20	23.36	2049.28	564.06	72.43	78.1244	13.87
-3.2	28.54	24.70	2049.28	591.14	75.91	78.1752	15.80

 $F = 1.0684$ ,  $N = 500$  ml,  $s = 0.566$  cal,  $W = 21.00$  g,  $m = 4.80$  g,  $T_{\Delta} = 0.10^{\circ}C$ 

Mean = 12.90

 $\sigma = 2.02$ PE<sub>m</sub> =  $\pm 0.56$ 

## DILATOMETER METHOD

Dilatometer No	Expansion 25 ml water on freezing	Factor	Expansion 25 ml gum arabic* on freezing	Bound water
	$ml$		$ml$	Per cent
1.....	2.47	0.0988	1.82	13.11
2.....	2.35	0.940	1.81	9.17
3.....	2.42	0.968	1.81	11.80
4.....	2.42	0.968	1.82	11.32
5.....	2.50	1.000	1.83	13.68
6.....	2.47	.0988	1.81	13.59

Mean = 12.11

 $\sigma = 1.74$ PE<sub>m</sub> =  $\pm 0.48$ 

\* 25 ml gum arabic, sp gr 1.042, contained 21.20 g of water and 4.65 g of gum

The mean values for bound water as determined by the three methods are in excellent agreement. The maximum difference is  $0.79 \pm 0.74$  per cent of bound water for the calorimeter and dilatometer methods. This is only about 6.5 per cent of the total bound water as measured by the dilatometer method and is without statistical significance in view of its probable error. The variability, as measured by the standard deviation ( $\sigma$ ), also was essentially the same for the three methods in these experiments. Too much reliability should not be placed on these values, however, as they were obtained from only six measurements. Refinements in the technic of the calorimeter method have been made since this comparison was finished. These have reduced the variation in repeated determinations.

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resistance, since the bound-water content near freezing temperatures is the item wanted, but in studying drought resistance this objection might be a serious one

### SUMMARY

No satisfactory definition of bound water can be made. All water that is not free water, that is, that does not show some of the common properties of liquid water, may be considered as bound water. In the methods of determining bound water, free water is measured and bound water is found by difference. This paper presents the results of a study of three methods of measuring bound water in plant tissue, namely, the cryoscopic method, the calorimeter method, and the dilatometer method.

The theory involved in the cryoscopic method is the assumption that bound water is not free to dissolve sucrose. A determination of the increased depression of the freezing point of the material after sucrose is dissolved in it will indicate whether or not all the water present is free for the solution of sucrose. The chief disadvantage of the cryoscopic method is that it can be used only for liquid or semi-liquid material. It is open to several other objections, the most important of which is that the addition of sucrose in molecular concentration to the material may change the bound-free water equilibrium or that the sucrose may be hydrolyzed or adsorbed by the material.

The theory involved in the calorimeter method is the assumption that bound water does not freeze. In this method the amount of water that changes to ice is determined by measuring the amount of heat necessary to thaw the frozen material. Owing to the great difference between the latent heat of fusion of ice and the specific heat of water, rather small quantities of ice can be measured. This method can be used on any kind of material, whether liquid, semiliquid, or solid.

The theory involved in the dilatometer method is also the assumption that bound water does not freeze. In this method the expansion of the material as freezing occurs is used to determine how much of the water changes to ice. This method can also be used on any kind of material, but great care is necessary to be sure that all air is removed from the material before it is frozen. Both the calorimeter and the dilatometer methods are open to the objection that the bound-free water equilibrium may be changed by the freezing of the material at the low temperature used.

The calorimeter method is recommended for the measurement of bound water in practically all materials, since it is easy, rapid, accurate, and reliable. Whenever possible, however, determinations should be made on the material by each of the several methods.

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# ELSINOE ON APPLE AND PEAR<sup>1</sup>

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## INTRODUCTION

This paper deals with the morphology and taxonomy as well as the history and distribution of *Plectodiscella piri* (55),<sup>2</sup> which causes an anthracnose of considerable importance, affecting apple (*Malus sylvestris* Mill.) and pear (*Pyrus communis* L.). Reference is made also to Sphaceloma, that is, the conidial stage of *Plectodiscella*, on other plants besides those named, most of which have not heretofore been reported as attacked by this group of fungi. *Plectodiscella* is shown to be a synonym of *Elsinoe*.

## HISTORICAL REVIEW

The history of *Plectodiscella piri* as then known was reviewed by Jenkins and Horsfall (31) in 1929, and the Sphaceloma or conidial stage of the organism reported. This was recognized on the basis of conidia on leaves of apple and pear from Transcaucasia,<sup>3</sup> part of the collection on which the perfect stage of the fungus was discovered, and on conidia in a theretofore unidentified culture isolated by Osterwalder (38) from a fruit spot on Jonathan apple grown in Switzerland. He diagnosed the disease as Jonathan spot, which is ordinarily considered to be nonpathogenic. A publication of Zschokke (57) in which he mentioned the occurrence of the disease on Jonathan apple, as referred to by Osterwalder (38), was not available to Jenkins and Horsfall in 1929. Now at hand, however, it reveals that not only the Jonathan variety (pl. 1, A), but several others there illustrated show dark lesions which appear to be those of the *Plectodiscella* disease.

In the past two years the writer has diagnosed the anthracnose on fresh apples intercepted in transit<sup>4</sup> from Ireland (fig. 1), Italy, Switzerland, and Hungary, and also on apples imported from Portugal. In each case isolations of the pathogene have been made. The apples from Ireland and Italy have been referred to in earlier publications (28, 52), those from Portugal, of the variety Reneta, were secured through the courtesy of Mathilde Bensaúde, of the Instituto Rocho Cabral, Lisbon. So far as the writer knows, this fungus has not been found in North America.

Although records are not available it is believed that *Plectodiscella piri* infects the twigs of apple and pear as well as the leaves and fruits. The larger lesions on the apple fruit shown in Figure 1 were brownish or

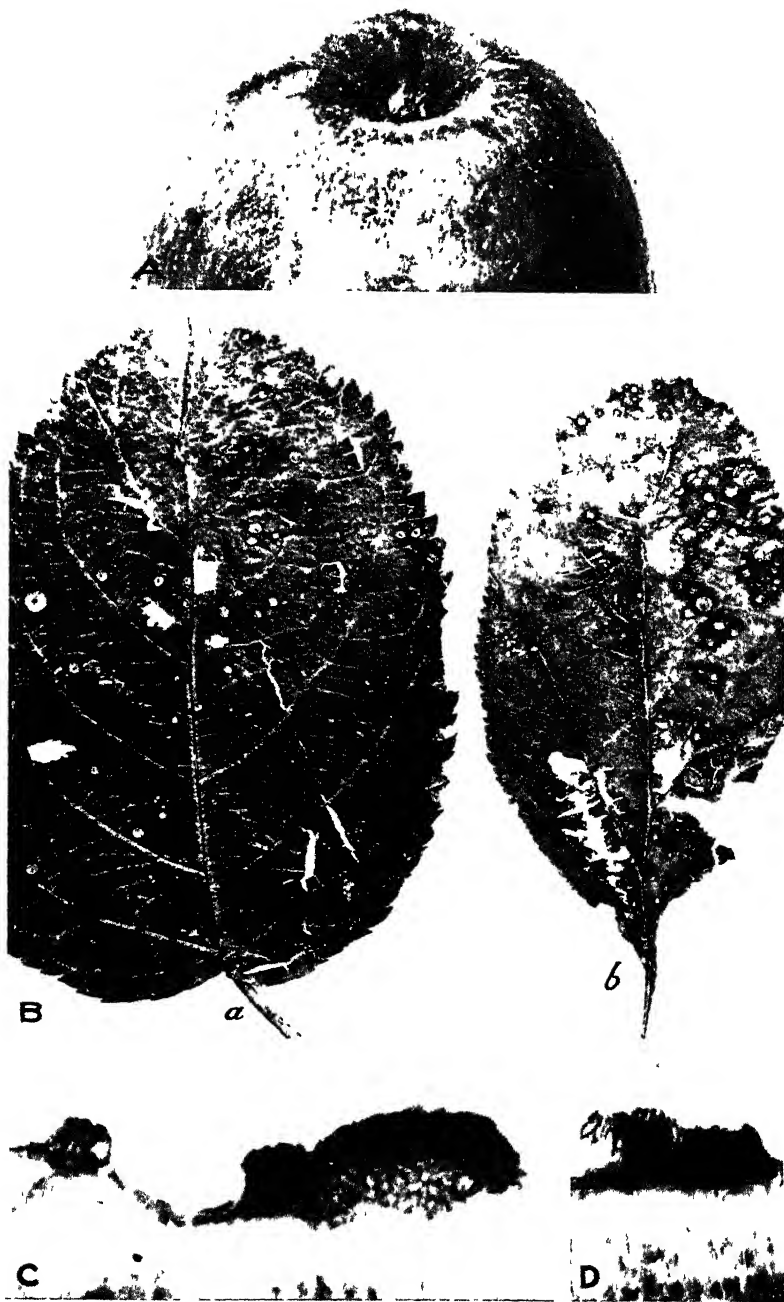
<sup>1</sup> Received for publication Dec. 8, 1931, issued May, 1932.

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<sup>3</sup> Russia, Transcaucasia, near Sotschi, Aug. 28, 1913. N. N. Woronichin. E. Herbarium, Institute of Mycology and Phytopathology, Leningrad, U. S. S. R. Contributed by N. N. Woronichin and A. Jacewski.

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A, Part of Jonathan apple fruit illustrated by Zschokke, showing lesions apparently of apple and pear anthracnose (X 1). B, *a* and *b*, general appearance of anthracnose lesions on upper surface of apple leaves (X 1). C and D, sections of conidial fructifications on upper surface of apple leaves (X 380). Material (B-D) from Woronichin, Transcaucasia, Russia, in 1913. Ex Herb. Inst. Mycol. and Phytopath. Leningrad. Photographs (A, C, and D) by J. F. Brewer and (B) by W. R. Fisher.

whitish at the center surrounded by carmine<sup>5</sup> or jasper red, while those on the other fruits examined were mostly madder brown or chestnut.

### MORPHOLOGY

Plates 1, B, *a* and *b*, and 2, A, show *Plectodiscella pyri* on apple and pear leaves from Transcaucasia,<sup>6</sup> on which conidia were found by Jenkins and Horsfall (31), and Plate 3, A and B, those of the fungus on pear leaves from Italy.<sup>7</sup> Although conidia were not abundant on the Transcaucasian material, they practically covered the acervuli examined on the Italian specimens (Pl 3, C and D). In all cases the conidia seen have been of the various shapes, sizes, and colorations

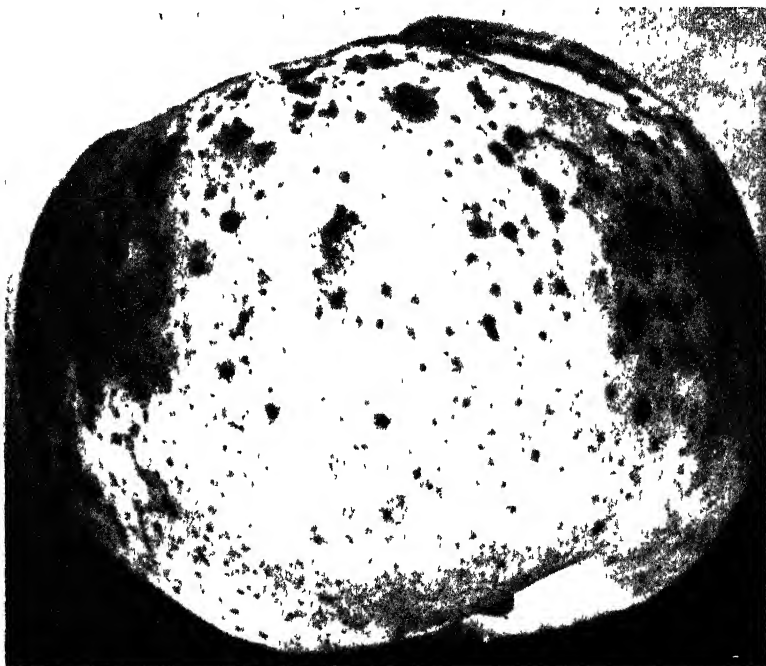


FIGURE 1—Apple fruit from Ireland affected by anthracnose. Slightly enlarged. Photograph by J. F. Brewer.

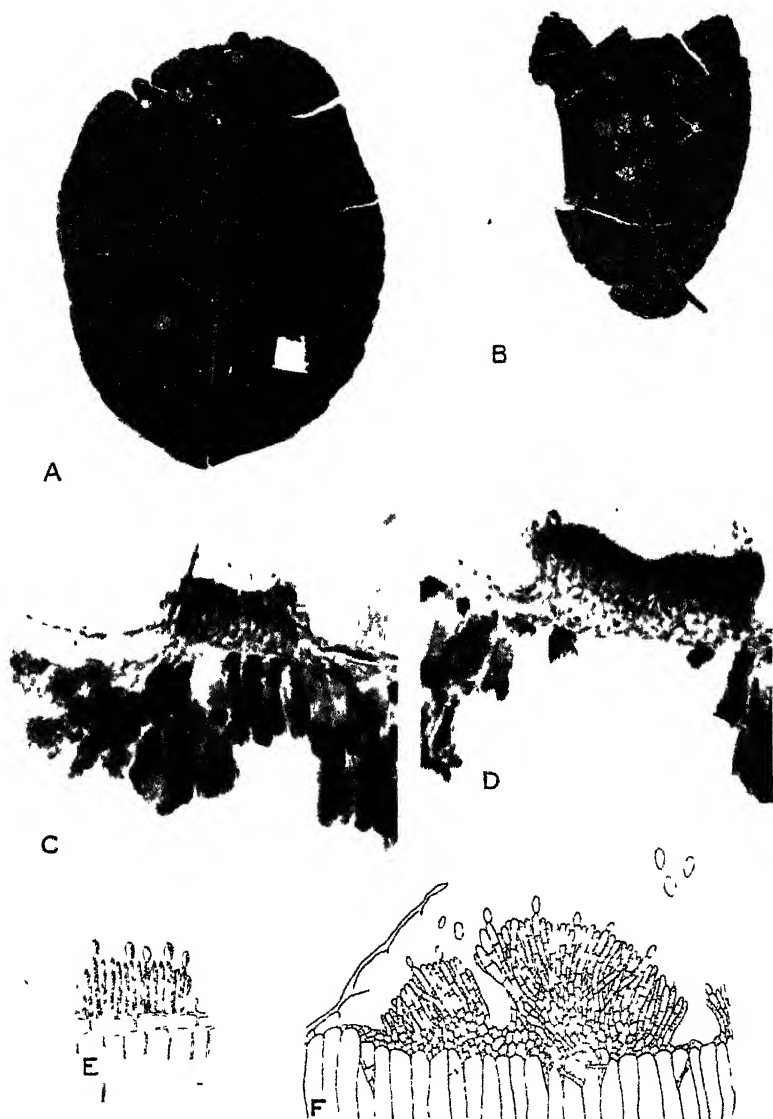
of those described for this fungus as referred to later in this paper (Pp. 4, 5). In addition, on one of the Italian specimens there was seen a 3-cell elongate colored body interpreted as possibly a greatly swollen conidium of this fungus. Hyaline conidia of the type illustrated in Plate 3, E and F, some of which were biguttulate, were produced in great abundance within an 18-hour period, when small masses of a young culture on potato-dextrose agar were transplanted to a corn-meal poured plate to which a few drops of sterile water

<sup>5</sup> Color reading by J. Marion Shull based on the following publication: RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., illus. Washington, D. C. 1912.

<sup>6</sup> Op. cit. (See footnote 3).

<sup>7</sup> BRIOSI, G. and CAVARA, F. I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OD UTILI. Fasc. 11, No. 274. Pavia, 1896. This material, labeled "*Hadrotrichum populi* Sacc.,"<sup>8</sup> consists of three specimens for which the following data are given: "Estate 1894—Sul Pero—Avelino—D. Peglion, id. 1891—id.—Orto botanico di Pavia, id. 1890—Sul Melo—id. id."

<sup>8</sup> This species was originally described on black poplar (*Populus nigra* L.) as doubtfully of the genus *Hadrotrichum*, and the binomial was therefore written as *H. ? populi* (13, p. 264). As in this instance, where the question mark has been dropped from this name in literature cited, it is also omitted in the present paper.



*Plectodiscella pyri*. A-D, On pear leaves from Italy, identified by Brioso and Cavares as *Hadrotrichum populi* Sacc and distributed by them as No 274, I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OP UTILI Fasc II Pavia, 1896. One of these leaf specimens of the fungus was collected at Avelino in 1894 by Peglion and is typical of his *Gloeosporium purinum*, the other was collected in 1891, at Pavia. A, Upper leaf surface ( $\times 1$ ), B, lower leaf surface ( $\times 1$ ), C and D, sections of acervuli on lesions on upper surface of A and of B, respectively ( $\times 380$ ). Photographs by J. F. Brewer. E, On pear leaves, as illustrated by Ferraris (19, fig 174, I), under the name of *Hadrotrichum populi*. F, On apple leaves, as illustrated by Maublanc (35, Figs. XIII and XIV), under the name of *Melanobasidium mali*.



Upper surface of pear leaves infected by *Plectodiscella pyri*. A, General appearance of lesions ( $\times 1$ ), B (enlargement of A, a), fructifications of both stages of the fungus ( $\times 22$ ). C-E, sections through fructifications represented in B. C, a, an acervulus. D and E, ascomata, with conidiophores at one side (a) of D. D, b, and E, a, asci. E, b, epithecium or dark stroma, covering ascoma. C,  $\times$  about 600, D and E,  $\times 380$ . Material from same source as that shown in Plate 1, B-D. Photographs by J. F. Brewer.

had been added. Some conidia were already present in the culture from which the transfers were made. Granular masses in some sections of lesions on apple fruits were interpreted as possibly consisting of microconidia.

Plates 1, C and D, and 2, C, *a*, represent the conidial stage of the fungus; Plate 2, D, both the conidial stage (*a*) and the perfect stage (*b*); and Plate 2, E, only the perfect stage. Woronichin's (55, fig 3, and pl. 1, fig 1) illustrations of dark hyphae at the apex of a young pustule of this fungus, as well as of converging hyphae at the center of another pustule on the same material, are interpreted by the present writer as conidiophores of *Plectodiscella piri*. These structures, however, together with such dark hyphae or stroma covering the ascoma as are here shown in Plate 2, E, *b*, are termed by Woronichin (55) the "epithecium" or "shield." The dark hyphae or stroma are believed to have formed entirely or almost entirely within the epidermis, as is illustrated for a closely related fungus on Lima bean (*Phaseolus lunatus macrocarpus* Benth.), tentatively identified as *Elsinoe canavahae* Rac. (30, pl 4, C, *c*, and pl 5, D and K, *b*). The Elsinoe structure illustrated in Plate 2, C, *a*, of the paper just cited seems to be a development homologous to what Woronichin (55, pl. 1, fig 6) described in *Plectodiscella piri* as a small, cup-shaped, almost superficial ascoma practically inclosed in a coat of brown cells. In both instances the darkening of the hyphae is possibly due to oxidation following exposure, or, as explained by Woronichin (55) in the case of the apple and pear fungus, to the pronounced isolation of the ascoma from the substratum.

It seems that since his report of the perfect stage of *Plectodiscella piri*, Woronichin (56, p. 221) has identified its conidial stage as *Hadrotrichum pirinum* (Pegl.) Sacc., which, as will be explained later, actually represents the conidial stage of this ascomycete. This identification by Woronichin was made in reporting a leaf spot on pear growing in a Caucasian nursery. His description of the lesions and of the disposition of pustules of the pathogene thereon is essentially the same as that given in connection with his (55) description of the perfect stage, i. e., *Plectodiscella piri*. The conidia are described as 4 $\mu$  to 5 $\mu$  in diameter (56, p. 221).

#### TAXONOMY

As stated earlier in this paper, Jenkins and Horsfall's (31) discovery of the imperfect stage of *Plectodiscella piri* was based upon conidia produced in a subculture of an isolation by Osterwalder and upon those found on the specimens from Transcaucasia on which Woronichin (55) had discovered the perfect stage. The recent finding of a Sphaceloma on an apple fruit from Italy (28) at once suggested that the fungus had long been known to Italian mycologists. The certainty of this is now established through the specimens from Italy identified by Briosi and Cavara as *Hadrotrichum populi* Sacc.<sup>9</sup> The fungus is here unquestionably of the genus *Sphaceloma*, and a comparison of it with typical material of *Plectodiscella piri*<sup>10</sup> shows that

<sup>9</sup> Op cit (See footnote 7)

<sup>10</sup> Op cit (See footnote 3)



Cette espèce est nettement parasite sur les feuilles du Pommier, où elle forme de petites taches blanches, bordées d'une marge subérisée. Je n'ai pas de renseignements sur l'extension de ce champignon et les spores de l'unique échantillon reçu n'ont pas germé

#### HADROTRICHUM PIRI

Acervulus punctiformibus, atris, in maculis foliarum dealbatis, epiphyllis, sub-superficialibus strato proligio subcuticolare, e cellulis oblongis dense stipatis fuliginis conflato, conidis globoso-ovoidies ( $4-5 \times 4\mu$ ), olivaceo-fuliginis

Hab In foliis vivis *Piri communis*, Montubeccaria (prov Pavia)

A few years before Peglion (39, p 6) described *Gloeosporium pirinum*, Cavara (17, p 184), reporting the occurrence of *Hadrotrichum populi* on *Populus nigra* L in Lombardy, Italy, stated that a closely allied form affects "*Rosa* esp cult" and "*Rubus corifolius*." He (18, p 282) later asserted that this *Hadrotrichum* species affects "*Rosa*, *Rubus*, *Sorbus*" and "*Pyrus*." At about the same time Briosi reported in Italy *Hadrotrichum* sp on wild rose (7, p xviii), and *H populi* (8, p xix) and *H populi* "forma del pero" (9, p xxv) on pear. He continued to refer to the pear fungus either as *H populi* (10, p 298, 12, p 663; 14, p 70) or as *Gloeosporium pirinum* (13, p 365). He also reported *H populi* on rose in Meaux, France (10, p 313), and on maple (*Acer negundo* L) (11, p 541) in Italy. Ferraris (20, p 875) included European mountain ash (*Pyrus aucuparia* Ehrh) among the hosts for *H. populi* var *piri*.

Even as late as 1920 Montemartini (36, p 122) was apparently unaware of the nomenclatorial status of his *Hadrotrichum piri*, for, citing only Cavara (18), he recognized this binomial and *Gloeosporium pirinum* as synonyms of *H populi*. At the same time he reported what he interpreted to be this fungus on pomegranate (*Punica granatum* L) growing at Montubeccaria, Pavia, the type locality for *H piri*.

More recently, Borg (5, p 233-239) and Marchionatto (33, p 10-11) have reported *Gloeosporium pirinum* on pear in the island of Malta and in Argentina, South America, respectively; while in the Crimea, Russia, Garbowski (21, p. 255-256) has identified *Hadrotrichum populi* on apple, pear, plum (*Prunus domestica* L), poplar (*Populus nigra*), and white beamtree (*Sorbus aria* Crantz), and, as previously noted, in the latter country Woronichin (56, p 221) has reported *H pirinum* on pear in the Caucasus and *Plectodiscella piri* on both apple and pear in Transcaucasia (55).

Garbowski (21, p 256) stated that in the Crimea *Hadrotrichum populi* causes considerable damage to fruit trees, particularly apple, he observed it on both leaves and fruits of several different varieties. He described the fruit spot as circular, with a whitish central part, and a reddish-brown border. Reaching a centimeter in diameter, the whitish area was dotted with the dark pustules of the fungus, which had ruptured the dead epidermis. He noted also the close resemblance of the fruit spot to that caused by *Phoma pomorum* Thuem., and stated that it is probable that infection due to the *Hadrotrichum* is often ascribed to this fungus.

Specimens of the so-called *Hadrotrichum* on apple leaves from the Crimea, as well as of *Gloeosporium pirinum* on pear from Argentina, recently received through the courtesy of Garbowski and Marchionatto, respectively, are of the same general appearance as those of

it is this species. The Italian material represents the fungus on pear leaves from Avellino and Pavia (pl 3, A-D) and on an apple leaf from Pavia. That on the leaf from Avellino is from the collection of *Gloeosporium pirinum* on which Peglion (39) based his description of that fungus.

The disease caused by *Gloeosporium pirinum* was termed "pear anthracnose" by Peglion (39, 40, p 267), and this name was later employed by Briosi (13, p 365, 14, p 70) in reporting the disease on pear. In the present paper the disease is referred to as anthracnose of both apple and pear. Peglion (39, 40, p 268) observed the similarity of the leaf lesions produced by this disease to those of grape anthracnose on leaves of grape (*Vitis*). He (39, 40, p. 268) made some notations on the susceptibility of pear varieties to infection by *Gloeosporium pirinum*. Recently he has written that this fungus is common in the region of Bologna, where he is now located.

Saccardo (44, p 136), in 1915, reporting the fungus on this host in France, made the new combination *Hadrotrichum pirinum* (Pegl), for which he gave the synonyms *Gloeosporium pirinum* Pegl (39, p 4), *Hadrotrichum piri* Montem (35, p 226), and *H. populi* Sacc var *piri* (Montem) Ferr (19, p 875). On the basis of Maublanc's technical description (34, p 70) and illustrations (34, figs xiii and xiv) of *Melanobasidium mali* Maub and an examination of a fragment of the specimen on which the description is based,<sup>11</sup> this fungus is here identified as the same as *Gloeosporium pirinum*, or *Plectodiscella piri*.

The reports of *Hadrotrichum pirinum* from France and of *Melanobasidium mali* from Spain constitute the only records at hand of *Plectodiscella piri* in these countries. Reference to its occurrence in Portugal was made in the historical section of this paper.

The illustrations of this fungus by Ferraris (20, fig 174, I), under the name of *Hadrotrichum populi*, and by Maublanc (34, figs xiii and xiv), under that of *Melanobasidium mali*, are reproduced in Plate 3, E and F, respectively, while the original technical descriptions of *Gloeosporium pirinum*, *Hadrotrichum piri*, *Melanobasidium*, and *M. mali*, typifying this genus, follow.

#### GLOEOSPORIUM PIRINUM Pegl

Maculis initio punctiformibus, rubro-cinctis, inde effusis, rotundis, saepe confluentibus, ad centrum griseis, vel sordide brunneis amphigenis, acervulis minutis 150-300 $\mu$  diam erumpentibus, olivaceo-chlorinis, conidiis ovatis vel subcylindraceis, continuis, eguttulatis, 6-4, hyalinis, basidiis bacillaribus, 20-25  $\times$  4, minute granulosis hyalinis vel dilute fumosis.

Hab. in foliis *Piri communis* prope Avellino.—It austr.—Vere 1894

#### MELANOBASIDIUM nov gen (Tuberculariées Dématiées).

Follicolum, maculicolum; sporodochia minima, erumpentia, atra, ex hyphis ramosis, septatis, intricatis composita, sporophoris cylindricis, densis, septatis, concoloribus vestita; conidia solitaria, acrogena, ovoidea, hyalina.

#### MELANOBASIDIUM MALI nov sp.

Maculis albidis, ovoides vel elongatis, margine brunnea, angusta cinctis, sporodochiis punctiformibus, epiphyllis, demum epidermide fissa superficialibus, 170-190 $\mu$  latis, conidiis ovoidis, hyalinis, 4.5-5.5  $\times$  2.5-3 $\mu$ .

In foliis vivis *Piri Mali* ad Sevilam, Hispaniae

<sup>11</sup> "Melanobasidium mali Maublanc sur *Prunus malus* Seville 1900" (fragment of type). Ex Herbarium, Station Centrale de Pathologie Végétale, Ministère de l'Agriculture, Institut de Recherches Agronomiques, Versailles, France. Specimen received through the courtesy of G. Arnaud.

One of the genera considered was *Elsinoe*, typified by *E. canavaliae* and originally reported on *Canavalia gladiata* (Jacq.) DC. According to Woronichin (55) the ascoma in *Plectodiscella piri* develops intra-epidermally, whereas in *Elsinoe canavaliae* it forms subepidermally; furthermore, lesions of scab of *Canavalia* are thickened, while in apple and pear anthracnose such lesions are not found. The two species can not be separated by these criteria, for intraepidermal as well as subepidermal ascomata occur in the Lima-bean fungus tentatively identified as *E. canavaliae*, furthermore, data at present available show that lesions resulting from attack by *Sphaceloma* may be not only of normal thickness, but also of increased or (3, 27) even of less than normal thickness. The fact that hyperplastic lesions occur in the anthracnose of brambles (16, 49) and of rose<sup>16</sup> suggests that they may be found in the apple and pear disease.

Woronichin (55) found resemblances between the perfect stage of *Plectodiscella piri* and *Mollerella* Wint (54, p 102) not *Moellerella* Bres (6, p. 292). The latter genus at the time his (55) paper was written had been removed (25, p. 349) from the family Myriangiacei Nyl (37, p 139) and then transferred (25, p 349) to the discomycetous family Agryiaceae Von Hohn (25, p 362). If more information had been available concerning *Mollerella* it is probable that Woronichin (55) would have classified his new fungus in this genus, which is older than *Elsinoe*. He (55) compared it with the myriangoid genera *Ascostratum* Syd and *Kusanoa* P. Henn, as well as with *Elsinoe* and *Myriangina* (P. Henn) Von Hohn (25, p 372-373), of these two it resembled *Elsinoe* more than *Myriangina*. *Myriangina* was originally classified by Hennings (24, p 55) as a subgenus of *Myriangium* Mont. and Berk (4), on which is founded the Myriangiaceae. Upon removing *Myriangina* from this family, Von Hohnel (25, p 373) erected for this genus, as well as for *Elsinoe* until then classified in the Exoascaceae (42), the family *Elsinoeaceae* Von Hohn. He was uncertain of its systematic position, but suggested that its affinities might be with the *Plectodiscaceae* or with the *Protodiscaceae*. Woronichin (55) concluded to place his new family *Plectodiscellaceae* between the *Elsinoeaceae* and the *Discomycetes*, explaining that it was undoubtedly related to *Mollerella*, and through *Ascostratum* and *Kusanoa* to the Myriangiaceae.

Soon afterwards the Myriangiaceae were treated as an order by Theissen (50, p 311), i. e., as the Myriangiales Starb (42), and the families *Elsinoeaceae* and *Plectodiscellaceae* were placed in this order by Theissen and Sydow (51, p 437). For these families they created the suborder Protomyriangiaceae and distinguished them by the presence of an epithecium or shield in the *Plectodiscellaceae* and its absence in the *Elsinoeaceae*. It has been shown earlier in the present paper that this is not a valid criterion for the separation of the type species of the genera *Elsinoe* and *Plectodiscella*; it follows, therefore, that it is not a valid criterion for the separation of the two genera or of the families created for them. In 1925 Arnaud (2) merged the two families and transferred *Elsinoe*, *Plectodiscella*, and *Myriangina* to Hennings's (22) genus *Uleomyces*. *Uleomyces*, *Myriangium*, *Kusanoa*, *Ascostratum*, and a few others fall in the Eumyriangiaceae of Theissen.

<sup>16</sup>JENKINS, A. E. Op cit (See footnote 12)

*Plectodiscella piri* from other sources, which have been examined by the writer.

Marchionatto (33, p 11), reporting the fungus only on leaves of pear, stated that it was fairly widespread in the Province of Buenos Aires and the islands of Delta del Parma. His advice (33, p 11) for the control of the disease is similar to that given by Peghon (39, 40, p 269).

The reports of *Hadrotrichum* on rose presumably concern the *Sphaceloma* on rose; those on brambles, *Plectodiscella veneta* (Speg.) Burk., or the perfect stage of *Gloeosporium venetum* Speg. (16), and those of *Hadrotrichum* and *G. pirinum* on apple and pear, *P. piri*. Although these three similar fungi, occurring on rosaceous hosts, and *Hadrotrichum* ? *populi*, on poplar, have been treated as comprising one and the same organism, it seems advisable to keep them, tentatively at least, as separate species, as originally described. It has been mentioned elsewhere by the present writer<sup>12</sup> that Alexander (1, p 72) reported infection of apple fruits by *P. veneta*, but that he did not furnish absolute proof of such pathogenicity. This species and the other two from rosaceous hosts just mentioned were separable, as far as compared by the writer,<sup>12</sup> but this is not interpreted necessarily to mean that each is entitled to the rank of species.

Landau (32, p 684), in referring to the doubtful classification of *Hadrotrichum populi* in the genus *Hadrotrichum* (43, p 264), suggested that the species be retained there until it could be investigated further. An examination of typical material of the fungus on *Populus nigra*,<sup>13</sup> on which, as previously stated, it was originally described, as well as of Briosi and Cava's<sup>14</sup> illustration of it, shows that it possesses the characteristics of the genus *Sphaceloma* and that it is possibly a distinct species. Therefore it is here transferred to the genus *Sphaceloma* as *S. populi* (Sacc), n. comb. The fungus is reported not only on black poplar, but also on Lombardy poplar (*P. nigra italica* DuRoi) in Italy<sup>15</sup> and South America (46, p. 192) and on "*Populus canadense*" (53, p. 305) in Italy.

Isolations of *Sphaceloma* from pear, poplar, or strawberry tree, or tests to determine whether the *Sphaceloma* on apple will infect pear, and that on pear apple, seem not to have been made, nor has there been available fresh material of the pear fungus or any specimen of what may prove to be *Sphaceloma* on maple, pomegranate, plum, European mountain-ash, or white beamtree.

Brizi (15) has reported an anthracnose of almond (*Amygdalus communis* L.) in Italy, which he regards as similar to anthracnose of grape; also Von Hohnel (26, p. 65-67) has added a species to Maublanc's genus *Melanobasidium*, but its characteristics as described (26, p 65-67) may exclude it from this genus.

Before erecting the ascomycetous genus *Plectodiscella* and the family *Plectodiscellaceae* for the apple and pear anthracnose organism, Woronichin (55) considered placing the species in one of several genera already described with each of which it has characteristics in common.

<sup>12</sup> JENKINS, A. E. ROSE ANTHRACNOSE CAUSED BY SPHACELOMA. (Unpublished manuscript.)

<sup>13</sup> Selva, Italy, October, 1877. (SACCARDO, P. A. MYCOTHECA VENETA, Century 8-12 (pr p) No 1256.) Cited in 43, p 264.

<sup>14</sup> BRIOSI, G., and CAVARA, F. I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OD UTILI. Fasc 13-14, No. 139. Pavia, 1900.

<sup>15</sup> Op cit (See footnote 14.)

## SUMMARY

*Plectodiscella piri* Wor, which causes apple and pear anthracnose, a disease of considerable economic importance, is widely distributed in Europe. It occurs also in South America, but is not known to be established in North America, although it has recently been intercepted at ports of entry in the United States.

The morphology, taxonomy, and history of the fungus are discussed. Structures termed the "epithecium" or "shield" by Woronichin in describing its perfect stage are interpreted as the conidiophores of its Sphaceloma or conidial stage. The data presented show that Woronichin's reasons for not placing this fungus in the genus *Elsinoe* are invalid, as is also Theissen and Sydow's basis for separating the families *Elsinoeaceae* and *Plectodiscellaceae*. Although Arnaud has transferred *Plectodiscella* to the genus *Uleomyces*, with consequent nomenclatorial changes, it is suggested that *Uleomyces* be investigated further before this transfer is accepted. *Elsinoe*, which Arnaud also treats as a synonym of *Uleomyces*, is here regarded as a distinct genus, and *Plectodiscella* and also *Melanobasidium* are considered as synonyms, *Plectodiscella piri* and *P. veneta* are referred to *Elsinoe*. *Melanobasidium mali*, the type of *Melanobasidium*, is regarded as a synonym of *Elsinoe piri*.

*Hadrotrichum populi*, on poplar, is transferred to the genus *Sphaceloma*.

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and Sydow (51), the only other suborder in the Myriangiales as classified by them (51). Arnaud, who (2) explains that the order is only imperfectly known, thus seems to disregard the two suborders of Theissen and Sydow. As a matter of fact, in the orientation of their stroma, Myriangina, as discussed by the writer (29) and by others, and some other related genera (48) actually partake of both suborders.

Arnaud (2, p. 678) transferred *Plectodiscella piri* and *P. veneta* to what he termed "the Elsinoe section" (2, p. 688) of the genus *Uleomyces*. Shear (45) did not agree with this transfer, because "*Uleomyces* has a superficial stroma and many-septate, muriform, brown ascospores, instead of 3-septate hyaline spores as in the type of *Plectodiscella*." Citing Arnaud's (2) account of *Elsinoe canavahae*, Shear (45) stated:

It is very clear that Elsinoe is a synonym of Plectodiscella, unless [here apparently following Theissen and Sydow (51) and not Woronichin (55)] the dark-colored superficial cover in Elsinoe be considered a distinctive character \* \* \*.

These statements were made by Shear (45) in connection with his report of *Elsinoe ampelina* (D By.) Shear, recently found by him in the United States, the fungus being possibly the same as *E. viticola* Rac. (31, 45), originally reported from Java.

As previously mentioned in the present paper, the writer (30) has shown that a superficial cover like that in *Plectodiscella* is found in *Elsinoe* on Lima bean. In other respects also the two genera are identical. Colored muriform spores occur in *Elsinoe canavahae* (30), so that in this particular *Elsinoe*, or *Plectodiscella*, agrees with *Uleomyces*. Colored ascospores occur also in the genus *Myriangium*, as already reported by Petch (41, p. 62-63). Had this characteristic of *Myriangium* been known to Hennings, according to his own statement, he (23) would have treated his genus *Uleomyces* as a synonym of *Myriangium*. Theissen (50, p. 312) and Theissen and Sydow (51, p. 439), on the other hand, separate *Uleomyces* and *Myriangium* on the basis of the distribution of asci in the ascoma, i. e., in *Uleomyces* the asci occur throughout the ascoma, whereas in *Myriangium* the lower part of the ascoma is sterile. Arnaud (2) recognizes the same distinction. Of these two genera only *Myriangium* has been cultured by the present writer, no living material of *Uleomyces* being available. It is evident that further investigation of *Uleomyces*, including a study of its growth in culture, is essential to a satisfactory comparison of this genus with the others just mentioned.

As previously stated, Woronichin (55) has suggested that *Plectodiscella* may be identical with *Mollerella*. It appears to be more closely related to this genus than to *Uleomyces*, but actual material of *Mollerella* is not available with which to make a direct comparison. The genus, which is older than *Uleomyces*, is included in the Myriangiales by Arnaud (2).

It is convenient here to treat *Elsinoe* as a distinct genus, with *Plectodiscella* and *Melanobasidium* as synonyms. *Plectodiscella piri* and *P. veneta* are referred to *Elsinoe* as *E. piri* (Wor.), n. comb., and *E. veneta* (Speg.), n. comb. In addition to *P. piri* and *Uleomyces piri*, synonyms of *E. piri* are, of course, *Gloeosporium pirinum*, *Hadrotrichum piri*, *H. populi* var. *piri*, *H. pirinum*, and *Melanobasidium malh.*

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# CANKER OF ASH TREES PRODUCED BY A VARIETY OF THE OLIVE-TUBERCLE ORGANISM, *BACTERIUM SAVASTANOI*<sup>1</sup>

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## INTRODUCTION

A canker of the European ash tree, *Fraxinus excelsior* L., occurs in England, Austria, Germany, France, and Italy. It is a stem disease of the canker type that progresses slowly from year to year, involving the bark and wood and stimulating the growth of the stem at the cankered areas. The disease has not been reported in this country. In 1922 the writer found what appeared to be bacterial cankers on a European ash tree in one of the parks in Washington, D. C. The branches of the tree were badly distorted with cankers, and the tree itself was nearly dead and was being cut down at the time. Some of the cankers were secured and from them numerous isolations were made, but no pathogenic organism similar to that obtained from European cankers could be isolated.

## REVIEW OF LITERATURE

Noack (2)<sup>2</sup> in 1893 described and pictured cankers on *Fraxinus excelsior* occurring in Germany. He saw bacteria while studying cross sections through the cankers, and concluded that the disease was a bacterial one, but he did not isolate the organism or produce the disease experimentally.

Vuillemin (8) claimed that the ash canker in France was the same as the olive-tubercle disease, but he did not give his reasons. He may have reached this conclusion because of the fact that the ash tree belongs to the same natural family as the olive; but there is no regular tubercle or knotlike appearance in the ash disease.

Massee (1, p. 520) described the ash canker as a distinct bacterial disease.

The 4- or 5-year-old stems or branches of young ash trees are frequently disfigured by the presence of cankered spots, varying in size from small cracks with thickened margins, half an inch long, up to rugged patches forming irregular cavities in the wood, and bounded by irregular outgrowths of callus, which may extend for several inches.

In a detailed account of the olive-tubercle disease, with a description of the olive-tubercle organism, *Bacterium savastanoi* E. F. Smith, Smith (6) included a paragraph about the ash-canker disease, stating that cankered stems had been sent to the United States from Europe and that from them the present writer had isolated an infectious organism that reproduced typical cankers on both American and European ash trees. At that time and for some years previously

<sup>1</sup> Received for publication Dec. 1, 1931, issued May, 1932.

<sup>2</sup> Reference is made by number (*italic*) to Literature Cited, p. 722.

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FIGURE 1—A, Ash canker from Vienna, Austria, in various stages of development. Photographed on arrival, December 8, 1913  $\times \frac{1}{2}$  B, Cross sections through ash stems, a, healthy, b, diseased. Natural size

Smith had been greatly interested in the olive-tubercle disease. He had investigated the disease and its causal organism, as well as the early literature connected with the subject (4, 5, 7). Although aware of the relationship of the olive and ash trees, Smith did not agree with Vuillemin (8) in the theory that both the olive-tubercle and the ash-canker disease were produced by the same bacterium. This difference of opinion, as well as the continued appearance of the olive-tubercle disease in the olive groves of California and the discrepancies in the tests of the Italian organism isolated from the olive tubercle by Petri and described by him (3), influenced Smith to send ash-canker material from Vienna and later olive tubercles from Portofino, Italy, to the writer in Washington, D. C.

### THE ASH-CANKER DISEASE

It was while Smith was in Europe in 1913 that he found ash trees badly diseased with cankers. One clump of trees, numbering about 40, in the neighborhood of Vienna, had more or less of the disease on both trunks and branches. Smith believed that by means of fresh isolations from the young ash cankers and from young olive tubercles the differences between the two organisms could be readily determined. Experiments carried out by the writer with both the Austrian and the Italian material convinced Smith that the ash-canker organism was a variety of the olive-tubercle organism, *Bacterium savastanoi*, and not a distinct species as he had supposed.

Material showing various stages of the ash canker was received in Washington a little more than a month after it had been sent from Austria by Smith (Fig 1, A and B.) The earliest stages showed a longitudinal split in the bark, from a few millimeters to a centimeter in length, with darkened raised tissue inside the split. The older stages showed a spreading out of this split, from which darkened corky tissue protruded. The disease did not often extend very far into the wood, the thickened portion being mostly bark, but the growth of the bark and wood was greatly stimulated, the diameter of the cankered area being often twice that of the stem above or below the canker. The largest cankers on the trees had not been secured; those received varied from 5 mm to 8 cm in length and from 3 mm to 5 or 6 cm in width. One stem 2.5 cm in diameter was almost encircled by cankers.

The disease disfigures the tree and destroys its commercial value. Noack (2) found instances in which leaves and leafstalks were infected. No diseased leaves were received by the writer, but since leaf infection of the olive tree is a common condition in the olive-tubercle disease, Noack is probably correct in his belief that infection may begin in leaves and leafstalks of the ash and spread to the stems.

Cross sections through the diseased portions of the cankers received from Vienna did not show bacteria in pockets like those in the olive stem. A white gummy substance that oozed from the sections was always present. This substance was filled with nonmotile particles which looked like bacteria but which because of their density could not be identified with absolute certainty. As the specimens were cut a long time before they were received, the condition of the tissues was somewhat changed and the bacteria were inactive.



FIGURE 2.—American ash stems inoculated with *Bacterium sarastanoi* var *frazini* isolated from European ash canker from Vienna, Austria. A and B, inoculated May 1, 1914, with two different colonies, of which B was the more infectious, and photographed August 10, 1914. C and D, control punctures on American ash stems made on the same date. About natural size.

## ISOLATIONS AND INOCULATIONS

Numerous sets of plates were poured, but no common colony appeared, probably because of the dry condition of the material. Various colonies were picked off and inoculations made into young American ash trees, *Fraxinus americana* L. Of nine colonies used, three proved to be infectious. Two of these are shown in Figure 2, A and B. The control punctures produced no outgrowth (Fig 2, C and D). The three colonies were alike in macroscopic appearance, all were white and resembled colonies of the olive-tubercle organism (*Bacterium savastanoi*)

European ash trees were inoculated with very satisfactory results (Fig 3, fig 5, C). The inoculum used was a subculture from an agar poured-plate colony. The infection took place more rapidly than on the American ash, and larger cankers, like the original ones from Austria, developed. In less than a week water-soaked areas showed around the inoculation pricks. A blisterlike swelling followed, then a split occurred. Later the curled bark and the roughened swellings of wood just under the bark extended for some distance out from the split. In five months cankers 2.5 cm long had formed. The splitting and darkening, with accompanying protuberances, continued to develop for a year or more until a large canker was produced.

In the spring the tender shoots of olive trees in the greenhouse were inoculated with subcultures of the original ash-canker organism. The shoots were watched far into the summer, but no well-defined canker or any indication of a tubercle appeared. Because of a slight disturbance in the tissue of the olive stem, it was thought for a time that cankers were forming, but the development did not continue. Inoculations on the olive were repeated twice with negative results (Fig 4, A and B).

Inoculations with an actively infectious strain of *Bacterium savastanoi*, isolated by the writer from olive tubercles received from Portofino, Italy, were made into both European and American ash in four different tests, but neither canker nor tubercle was produced. Photographs of ash stems, some inoculated with the olive-tubercle organism and others with the ash-canker organism, are shown in Figure 5, A and B.

The virulence of this isolation of *Bacterium savastanoi* had been previously established by repeated successful inoculations and was now tested again on olive trees. In two months knots 2 to 2.5 cm in diameter had grown at the inoculated places (Fig 4, C and D).

The ash-canker organism was reisolated from the cankers produced on the inoculated European ash trees, and this reisolated organism likewise produced typical cankers when reinoculated into other European ash trees. (Fig 6, B, and fig. 7). Control punctures are shown in Figure 6, A.

The cankers produced by inoculations were studied in cross sections stained and unstained, as the original European material, which was received a long time after it was cut, had been found unsatisfactory for section study. The bacteria were abundant between the cells of the cork and the bark parenchyma and also in a slime in the cavities formed by the disintegration of the cells. Here they were seen to be motile. There were no pockets, however, as in the olive tubercle. That bacteria were present in the wood also was proved by isola-



FIGURE 4—A and B, Olive stems inoculated with the ash-canker organism October 31, 1914, and photographed February 18, 1915, C and D, olive stems inoculated with the olive-tubercle organism December 4, 1914, and photographed February 18, 1915. All natural size



FIGURE 3—European ash stems inoculated with ash-canker organism May 1, 1915, more than a year after isolation from European material. Photographed October 19, 1915 X nearly 2



FIGURE 6—European ash stems inoculated with a reisolated colony of the ash-canker organism. A, Controls punctured with a sterile needle. B, stems inoculated with reisolated colony July 13, 1914, photographed August 10, 1914. All natural size.

tions, as the organisms could not with certainty be determined microscopically

#### LABORATORY TESTS AND COMPARISONS OF ASH-CANKER AND OLIVE-TUBERCLE ORGANISMS

Although *Bacterium savastanoi* and the ash-canker organism did not cross-inoculate in any of the experiments, cultural and morpholog-



FIGURE 5 — European ash stems inoculated with the olive-tubercle organism and the ash-canker organism. A, Stem inoculated with the olive-tubercle organism April 30, 1915, and photographed May 12, 1915. Neither tubercles nor cankers formed.  $\times 5$ . B, Stem inoculated with the ash-canker organism April 30, 1915, and photographed May 1, 1915.  $\times 5$ . C, Stem inoculated with the ash-canker organism May 7, 1915, and photographed October 10, 1918. Natural size.

ical tests showed them to possess a marked degree of similarity, and it is thought that they must be closely related species or varieties of the same organism exhibiting individual host tendencies. Comparisons were made, not only with the usual laboratory tests but also with the tests which Petri (3) used in his study of *Bacterium savastanoi* and which he published in 1909. (Tables 1 and 2.)

TABLE 1.—Comparison of the olive-tubercle (*Portofino strain*) and the ash-canker organisms by general laboratory tests

Specification	Ash-canker organism	Olive-tubercle organism ( <i>Portofino strain</i> )
Colonies on beef-agar plates.....	Surface colonies smooth, flat, glistening, white, mostly circular, some with entire, some with erose margins. Margins bluish in transmitted light, colonies translucent white in reflected light. Diameter 3 mm in 3 to 4 days at 25° C.	Same as ash-canker organism, except no erose margins, and bluish margins wider in transmitted light
Beef-agar slants.....	Margins faintly undulate, growth rather heavy, translucent white spreading.	Same as ash-canker organism, except growth not so heavy and surface a little smoother
Beef-infusion bouillon (Witte's peptone).	Clouded throughout at second day. No pellicle or rim at 4 days (room temperature 28° to 30° C). Grows better at 25° than at 30° 8 to 10 days' growth heavier at surface, becoming an incomplete pellicle	Same as ash-canker organism
Peptone water (Witte's) 2 per cent. solution	Heavy growth with pellicle.....	Do
Fernin's solution.....	Fine white growth with pellicle, which breaks up into floccs and filaments.	Do
Uchinsky's solution.....	Growth white, fine clouding throughout. No color change, incomplete pellicle, which breaks up on handling the tube. A viscid swirl rises from bottom of tube on shaking	Do
Cohn's solution.....	Fine white growth throughout, crystals at surface. Old flask cultures show a slight green tinge in the medium.	Same as ash-canker organism, except old flask cultures show no green color
Bouillon over chloroform.....	Growth fair but retarded.	Same as ash-canker organism
Tolerance of NaCl in beef bouillon.....	Good growth on bouillon containing 0.5 per cent, no growth in that containing 1, 1.5, or 2 per cent	Do
Beef-gelatin plates.....	Beef-infusion gelatin at 14° C shows no liquefaction of colonies in 34 days. Then flat, circular, translucent colonies develop with undulating margins. Rugose. Diameter 2 to 3 mm in 6 days, 4 mm in 11 days	No liquefaction. Margins undulating. Fine internal striae present. Colonies 1.5 to 2.5 mm in diameter at end of 4 days, white, circular, flat, thin
Beef-gelatin slabs.....	Slight surface growth at 14° to 16° C in 5 days, flat, thin, translucent. Faint growth down stab in 17 days. No liquefaction	Same as ash-canker organism, except white rather than translucent
Starch jelly.....	Cream-colored growth in 4 to 9 days. Surface growth yellowish in 31 days, medium pearl-gray at bottom of tube	Growth white. Instead of cream-colored in 4 to 9 days. In 31 days surface growth and medium the same as for ash-canker organism
Litmus milk.....	Begins to blue in 4 days, gradually becomes dark blue. No coagulation.....	Same as ash-canker organism, except color change a little slower.
Sterile milk.....	No change noticed until 20 days, when the milk becomes tan color, deeper than control. No coagulation. Does not form acids in milk, does not coagulate by a lab ferment	Do
Gas formation and behavior in fermentation tubes containing 2 per cent of Witte's peptone and 1 per cent of carbon compound as follows	No gas produced in any of the carbon compounds.....	Same as ash-canker organism
Mannite.....	Heavy growth in open arm of tube, medium dark brown, no rim, no pellicle, neutral litmus paper unchanged	Same as ash-canker organism, except medium not so dark and small clumps of pseudozooeciae on the surface
Glycerin.....	Heavy growth in open arm and heavy precipitate, rim, medium darkened, neutral litmus paper unchanged.	Same as ash-canker organism
Cane sugar.....	Medium, dark brown, heavy growth and a heavy precipitate; rim. Growth continued two-thirds of the way around U of the tube. Faint acid reaction to neutral litmus paper	Same as ash-canker organism, except medium light brown and clumps of pseudozooeciae on surface



FIGURE 7—European ash inoculated July 13, 1914 with organism reisolated from a canker produced on American ash, photographed October 10, 1918. Note cankers forming on small stem. Inoculations were made only on large stem. A little less than natural size.

Tolerance of acids in neutral beef bouillon containing Malic acid.....	No growth in medium containing 0.5 per cent or 0.2 per cent, pH 4.9. Good growth in medium containing 0.1 per cent, pH 6.2. No growth in medium containing 0.2 per cent, pH 4.7.	Same as ash-canker organism Do
Hydrochloric acid.....	Good growth in medium containing 0.1 per cent, pH 6.8, and fair growth in medium containing 0.2 per cent, pH 5.6.	Same as ash-canker organism except no growth in medium containing 0.2 per cent hydrochloric acid, pH 5.6
Production of indol.....	No indol in 10 days, slight amount in 22 days.	No indol in 10 days, slight amount in 21 days
Hydrolysis of starch.....	Organism streaked on starch-agar plates and grown for 5 days. Surface of plate flooded with saturated solution of iodine in 50 per cent alcohol. Medium showed destruction of starch extending $\frac{1}{2}$ to 1 cm on each side of growth streak.	Under same treatment as in ash, starch destruction greater. Zone of starch destruction extended $\frac{1}{2}$ to 2 cm on each side of growth streak
Production of hydrogen sulphide.....	None.	None
Nitrate reduction.....	Tested at 10, 16, and 20 days. No reduction to nitrites.	Same as ash-canker organism
Relation to light as shown by sunlight test.....	Tests made at noon in April, August, and October with thinly sown, beef-agar plates on ice bases bottom up, one-half plate exposed to sunlight. Colonies appeared on plates exposed 5 minutes but not on those exposed 10, 20, or 30 minutes. Colonies appeared on covered side of plates. Temperature of ice bag did not exceed $15^{\circ}$ to $19^{\circ}$ C.	Do
Temperature relation.....	Optimum, $25^{\circ}$ to $28^{\circ}$ C.; maximum, $42^{\circ}$ , minimum, $5^{\circ}$ . Thermal death point between $43^{\circ}$ and $46^{\circ}$ when fresh-beef-bouillon cultures were exposed in water bath for 10 minutes.	Optimum, $25^{\circ}$ to $28^{\circ}$ C.; maximum, $34^{\circ}$ to $38^{\circ}$ , minimum, $12^{\circ}$ . Thermal death point same as in ash-canker organism
Relation to acid and alkali.....	In beef-infusion bouillon the pH range is 6.0 to 8.5 with optimum 6.8 to 7.0.	In beef-infusion bouillon the pH range is 6.5 to 9+, with optimum 7.0 to 7.2
Relation to oxygen.....	Organism an aerobe. Tested in fermentation tubes with various media, also in shake cultures made with beef infusion-peptone agar containing dextrose.	Same as ash-canker organism
Chromogenesis.....	On beef agar, white, on beef gelatin, translucent white, on potato cylinders, olive-buff after 7 days' growth.	On beef agar and beef gelatin, white, on potato cylinders, deep olive-buff after 7 days' growth.
Morphology.....	Short rods, in culture media, single, in pairs, or in short chains, 1 to 4 $\mu$ long. flagella. Rods stained with carbolfuchsin $1.2\mu$ to $3.3\mu \times 0.4\mu$ to $0.8\mu$ (2-day beef agar culture). No spores.	Rods same as in ash-canker organism, except for a slight difference in size when grown on beef agar. 2 days stained the same, $1.2\mu$ to $1.9\mu \times 0.4\mu$ to $0.5\mu$ (2-day beef-agar culture), chains sometimes longer than in ash. Flagella same as in ash-canker organism. No spores.
Staining reactions.....	Stained readily with carbolfuchsin, methyl violet, and dahlia. Gram-negative and not acid-fast.	Same as ash-canker organism
Index number.....	5 020-31, 90 <sup>b</sup> -1, 2 <sup>c</sup> , 2	Do

<sup>a</sup> Witte's peptone was used throughout this test.  
<sup>b</sup> The colors mentioned in this paper are according to Ridgway (Ridgway, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 4<sup>th</sup> ed. illus. Washington, D. C. 1912.)

TABLE 1.—Comparison of the olive-tubercle (*Portofino strain*) and the ash-canker organisms by general laboratory tests—Continued

Specification	Ash-canker organism	Olive-tubercle organism ( <i>Portofino strain</i> )
Gas formation and behavior in fermentation tubes etc.—Continued		
Galactose	Heavy growth in open arm, heavy precipitate and rim. Clumps of pseudozoogloae at surface. Color of medium unchanged. Acid reaction to neutral litmus paper.	Same as ash-canker organism
Dextrose	Heavy growth with clumps of pseudozoogloae at surface, heavy precipitate, medium darkened slightly. Acid reaction to neutral and blue litmus paper. Liquid in open arm tested pH 6.6.	Same as ash-canker organism, except liquid in open arm tested pH 6.6
Lactose	Heavy white precipitate in wortlike strand, white rim. Medium darkened. Neutral litmus paper unchanged.	Same as ash-canker organism
2 per cent peptone water only	Good growth, moderate amount of precipitate, faint rim. Medium darkened slightly. Neutral litmus paper unchanged.	Do
Litmus-peptone agar	Slight growth in 10 days. No color change in 4 weeks.	Do
Litmus-peptone-dextrose agar	No color change in 10 days.	Do
	In 19 days medium reddened.	Do
	In 29 days no further change.	Do
	In 49 days still only moderate growth, and red color dulled.	At 67 days medium still a good red color
	In 10 days medium of all cultures partly red, others unchanged.	In 10 days medium of all cultures red
	In 19 days medium of all cultures red.	In 67 days medium still red
Litmus-peptone-galactose agar	In 67 days medium of 2 cultures blue and of 1 purple. Moderate growth of organism.	
Litmus-peptone-saccharose agar	Good growth readily.	Good growth readily
	In 10 days medium of all cultures a faint purple.	In 10 days medium of all cultures distinct purple
	In 19 days medium part red and part purple.	In 19 days medium purple—no red
	In 29 days the medium of the various cultures varied from red to purple.	In 29 days medium purple—no red
	In 67 days medium varied from dull red to dull purple.	In 69 days medium all dull purple
Litmus-peptone-glycerin agar	Good growth of organism in 10 days and no color change.	No color change in 10 days, good growth
	In 19 days no color change.	In 19 days medium a faint purple
	In 29 days a trace of blue in medium.	In 29 days medium a muddy purple
	In 67 days no further change.	In 67 days no further change
Litmus-peptone-lactose agar	Moderate growth of organism in 10 days.	Same as ash-canker organism
	In 19 days medium unchanged.	Do
	In 29 days medium still unchanged.	Do
	In 67 days medium blueed a little.	Do
Litmus-peptone-maltose agar	Moderate growth in 10 days and no color change.	Do
	In 19 days medium had blueed slightly.	Do
	In 67 days no further change.	Do
Litmus-peptone-mannite agar	Good growth and no color change in 10 days.	Good growth and medium red in 10 days
	In 19 days medium had changed to a dull purple.	In 19 and 29 days medium still red
	In 29 and 67 days medium still a dull purple.	In 67 days medium a dull purple

Steamed potato Test 1	Colony viscous, threadlike, pearl white. Starch transformed to maltose and amylo- dextrin. Substratum not browned with age of culture.	Same as ash organism.	Growth in 3 days, pale olive-buff, viscid, medium slightly grayed. Not tested for maltose and amylo-dextrin.
Test 2			In 6 days, medium brownish gray, in 25 days, wood-brown. 8-day cultures Mashed up with water, 2 cc of strong iodine in alcohol gave blue color not so deep as control, no purple. Color faded entirely in 5 minutes, 1 cc iodine added, color faded out again immediately. 1 cc iodine added, color remained same shade as <i>Bact. savastanoi</i> (Fortolino) for half an hour, then gradually grew lighter. Control retained blue color.
Steamed carrot	White, pearllike, salient, nonviscid growth.	No growth in 3 days Very faint white growth in 4 days In 6 days, growth still faint, not viscid, medium unchanged. In 25 days, a heavy white precipitate, growth still thin, medium unchanged. Test 1 No growth. Test 2 No growth.	Growth thin, white, in 3 days. In 6 days, growth not abundant but heavier than <i>Bact. savastanoi</i> (Fortolino), not viscid, medium unchanged. In 25 days, a heavy white precipitate, growth still thin, medium unchanged. Test 1 No growth. Test 2 No growth.
Bile-salt agar (sodium-gly- cocolate, 2 g., sodium taurocholate, 2 g., agar, 10 g., water, 100 g.) Cohn's solution	Good development Colonies round and white. Clouding after 48 hours at 15° C. At 24° map- precitate hastening of growth. Bacteria united into a rim, elongated, motile, slender. After a month, abundant accumulation of crystals of double phosphate of ammonia and magnesia.	Same as ash-canker organism up to eleventh day days, growth was not so heavy as ash organism, but there were more crystals. In 81 days, no color change. Bacteria motile, elongated forms abundant, wine (thin).	In 3 days, fair amount of growth. In 5 days, thin pellicle with crystals, fine white growth throughout. In 11 days, no difference in ash-canker organism than <i>Bact. savastanoi</i> (Fortolino), many crys- tals floating on surface, fine white growth throughout. In 40 days, rather heavy clouding, but not many crystals. In 81 days, faint tinge of green. Crystals still not abundant. Bacteria motile, a few elon- gated forms, no chains. In 5 days thin pellicle, coarse floccs, no crystals In 11 days rim, incomplete pellicle, crystals on surface. In 21 days pellicle still thin, rim present, many crystals at surface, largest not more than 1 mm in diameter.
Cohn's solution, 100 g., an- hydrous dextrose, 1 g.	Growth very rapid and abundant, thick pellicle consisting of rods united into bunches, many motile and wavy crystals (1-1.5 mm long) on pellicle and along walls of tube. In about 20 days superficial strata of liquid, color of green peas but lighter. Abundant white precipitate in bottom of tube.	In 5 days coarse floccs, no pellicle, no crystals. In 11 days no rim, faint almost imperceptible pellicle made up of crystals. In 21 days no rim, no pellicle except floating crystals about 1 mm in diameter. Bacterial growth not heavy, rods united into bunches, precipitate.	Bacterial growth in pellicle made up of rods united into bunches, precipitate, no green color in medium. In 28 days one ash culture had a faint trace of green. In 80 days decided green-pea color in all 3 cultures.
		In 28 days no green color. In 80 days still no green color.	

TABLE 2.—Comparison of cultural and physiological tests of the olive-tubercle organism (*Portofino strain*) and of the ash-canker organism with Petri's tests of the olive-tubercle strain

[Petri's formulas were followed]

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Nutrient agar (Witte's peptone, 1 g, anhydrous dextrose, 1 g, agar-agar, 1.5 g, distilled water, 100 g)	Colonies visible under microscope (X50) after 24 to 48 hours if bacteria came from another culture, after 3 to 6 days if they came from the tubercles or from the olive fly (temperature 17° C.). Maximum diameter, 2.2 μ to .5 μ. Colony round, white, translucent, then opaque, entire margins.	Colonies up in 48 hours, very small, round, translucent white, entire margins, thicker than ash colonies	Colonies up in 48 hours, translucent white, but many not round, erose margins 2 to 3.5 mm in diameter
Nutrient agar (Marek's dry peptone, 1 g, dextrose, 1 g, agar-agar, 1.5 g, distilled water, 100 g).	Slant cultures Extended milk-white growth, margins undulate and a little raised. Not viscid	Slant cultures Colonies massed together making an uneven, thick, corrugated surface, almost opaque, thicker and heavier than ash colonies, in 6 days flattened out and spread somewhat. Not viscid	Slant cultures Minute colonies, rather opaque, in 6 days fused somewhat and surfaces less corrugated. Not viscid
Nutrient agar (decoction of young branches of olive neutralized, 100 g, cane sugar or dextrose, 1 g, agar, 1.5 g).	After 60 hours, first colonies noted under microscope, growth soon arrested.	No colonies-----	No colonies
Nutrient agar (decoction of young branches of olive neutralized, 100 g, cane sugar or dextrose, 1 g, agar, 1.5 g).	Slant cultures Poor development, soon ceasing. Inoculations often sterile	No growth on slant agar-----	No growth on slant agar
Nutrient gelatin (Witte's peptone, 1 g, dextrose, 1 g, gelatin, 10 g, distilled water, 100 g).	Slow development. Colonies small, white. Tannic substances an obstacle to development	No growth-----	No growth
Bean agar (broth of beans, neutralized, 100 g, agar, 1.5 g)	Small white translucent colonies on plates-----	Test 1 Colonies up in 11 days, small and white, most of them round, a few indented	Test 1 Colonies up in 6 days, very tiny, in 12 days, largest 1.5 mm in diameter, white and deeply indented at margins
	Slant culture Growth similar to that on dextrose-peptone agar	Test 2 Colonies up in 7 days, under hand lens, some with fringed growth at margin, others erose, like ash colonies	Test 2 Colonies up in 3 days, pin point, in 7 days, only 1 mm in diameter, white with darker centers, erose margins, only a few round or nearly round
	Stab culture Scanty development in canal, colony at surface round, no liquefaction	Stab cultures Test 1 No growth. Test 2 Tiny white opaque colonies on surface at 20 days, feathery development in canal, no liquefaction	Stab cultures Test 1 Growth after 6 days. Test 2 Growth after 3 days, on surface, small colonies, white, opaque, irregular margins, midway, small bunch of colonies very fine and clustered, no liquefaction
	Substratum in which degeneration of the bacteria was much retarded from 4 to 6 months. Effect increased by addition of trace of sodium or potassium phosphate	Colonies grew well, but not so large as ash, granular markings, not seen on surface	Good medium for this organism. Colonies on plates not always round, erose margins. Twice the size of <i>Bact. savatanoi</i> (Portofino)
		Slant cultures, good growth, very like beef agar	Slant cultures, good growth, very like those on beef agar

Whey of milk with litmus (The curd was precipitated with dilute hydrochloric acid, then the whey was neutralized by adding sodium hydroxide, litmus added gave a pale lavender color)	No acid produced	Same as ash organism, except at 3 days the color of medium was a little deeper blue	Good growth in 3 days, medium slightly bluish. Not so deep a color as <i>Bact. senftenbergii</i> (Portofino). In 8 days medium forget-me-not blue and produced	No
Witte's peptone, 1 g., sodium chloride, 0.5 g., distilled water, 100 g., rosolic acid (0.5 per cent solution), 1 g.	In 3 days color changed to yellowish (production of acid) In 15 days return of reddish color (production of alkali)	Good growth. Slight change in color of medium in 3 days, paler than control. In 9 days medium several shades lighter than control but never yellowish. In 2 months medium darker than control, same color as ash organism. Tested with litmus paper when cultures were 3 months old. All gave an alkaline reaction. Controls faintly acid.	Good growth, color never became lighter	
Carrot sterilized at a low temperature to test for production of cytochrome	The prolonged development did not cause disintegration of cells, apparently no production of cytochrome. In certain such cultures was inactive on middle lamellae of cell walls of sections of fresh carrot	Same as ash organism. Cytochrome not developed	In 2 months medium several shades darker than controls, same as <i>Bact. senftenbergii</i> (Portofino). Tested with litmus paper when cultures were 3 months old. All gave an alkaline reaction. Controls faintly acid. 64-day-old cultures mashed and juice extracted. Raw carrots washed carefully, then immersed in litmus (1:1000) for 5 minutes and rinsed in sterile water. Slices placed in deep Petri dishes and juice poured on the cut surfaces. No change in 10 days	
Cohn's solution, 100 g., potato starch, 0.5 g.	After 2 or 3 days starch transformed to maltose and amyloextrin. No active amylase evident from the cultural liquid	Same as ash organism up to 5th day, then showed more and larger crystals	In 3 days, white growth near surface of liquid. In 5 days, thin pellicle and fine white growth throughout. Crystals in pellicle and on surface of starch layer at bottom of tube.	
Witte's peptone, 1 g., sucrose, 2 g., water, 100 g.	Sample of cultural fluid tested with Fehling's fluid. After 8 days' growth another sample of the organism showed a certain quantity of glucose. The cultural fluid passed through a Kieselguhr filter, with thymol and 0.5 per cent saccharase added, showed presence of invertase.	Same as ash organism.	After 8 days' growth, culture tested with Fehling's solution and glucose found. Greater reduction in ash organism than in <i>Bact. senftenbergii</i> (Portofino). Some cultures killed by heating at 55° C. for 20 minutes. Allowed to stand for several days, then transfers made to see if cultures were alive. No growth in transfers in 4 days, so heated cultures were considered dead. A crystal of thymol was added to each culture and 0.5 per cent saccharase. No growth in 3 days, when test showed the presence of invertase.	
Dunham's solution for production of indol	A culture 20 days old showed a reddish color. Production of indol not very abundant	21-day-old cultures, tested with sulphuric acid and sodium nitrite (fresh solution) gave a pink color without heating. Deep vinaceous	21-day-old cultures tested for indol with sulphuric acid, sodium nitrite (fresh solution) gave a pink color after heating, but not so deep as <i>Bact. senftenbergii</i> (Portofino). Vinaceous	

TABLE 2—Comparison of cultural and physiological tests of the olive-tubercle organism (*Portofino strain*) and of the ash-canoker organism with *Petri's* tests of *has olive-tubercle strain*—Continued

Medium	Olive organism ( <i>Petri's</i> Italian strain)	Olive organism ( <i>Portofino</i> strain)	Ash organism ( <i>Vienna</i> strain)
Witte's peptone, 1 g, anhydrous dextrose, 1 g, glycine, 2 g, water, 100 g	Rapid clouding. Mottled and elongated forms. Interrupted pellicle consisting of a hyaline mucous substance apparently from degeneration of bacterial membranes.	Mucous pellicle in 4 days, uniform growth below. Pellicle under microscope has mottled but no elongated forms. Definite threads (protoplasma) sometimes connect one another. Threads sometimes free from bacteria, but bacteria sometimes drift out nearly to a point by thread. Vegetative forms also in pellicle mass. Some forms rounded a little at extremities.	No pellicle, clouding not so heavy as in <i>Bact. sacchara</i> ( <i>Portofino</i> ). Mottled but no elongated forms. No gelatinous threads.
Mere's peptone from flesh, 1 g, anhydrous dextrose, 1 g, glycerol, 2 g, water, 100 g	In 3 days, at 25° C., liquid still clear. In 4 days almost unappreciable growth visible to naked eye. In succeeding days, clouding still very slight.	No growth.	Some forms rounded a little at extremities. No growth.
Neutral beef bouillon with Witte's peptone	Growth not abundant, thin white pellicle.	Same as ash organism.	Growth faint up to eighth day, then heavier, abundant by sixteenth day. No rim or pellicle.
Uchinsky's solution	Moderate development, less than in Cohn's solution, bacteria mottled and elongated.	Faint growth in 3 days, a trifle more than in ash organism. In 11 days no rim or pellicle. In 19 days growth still slight. Bacteria mottled and elongated 4 to 10 times usual length. In 21 days same as ash organism. In 48 days no further change.	Faint growth in 4 days. In 11 days no rim or pellicle. In 19 days growth still slight. Bacteria mottled and elongated 4 to 10 times usual length. In 21 days growth still faint and less than in Cohn's solution. In 48 days no further change.
Sterile milk	Good development. Milk not coagulated. After 20 days, reaction neutral.	Same as ash organism.	No change noted until seventeenth day, then indication of clearing. In 25 days milk entirely cleared without coagulation. In 30 days, milk pale olive buff. Reaction slightly alkaline to litmus, nearly neutral to phenolphthalein.
Test for reduction of nitrates (Witte's peptone 1 g, nitrate of potash, 0.3 g, distilled water neutralized with sodium hydroxide, 100 g)	No reduction of nitrates.	No reduction of nitrates.	No reduction of nitrates.

Xylose, 3 per cent.-----	do.-----	Test 1 No growth. Test 2 No growth.	Test 1 No growth Test 2 No growth in 6 days, faint growth in 12 days, still faint in 4 weeks
Lactose, 3 per cent.-----	Scarcely appreciable development.	Test 1 No growth. Test 2 No growth.	Test 1 No growth Test 2 Trace of growth in 5 days, no heavier in 4 weeks
Merc's peptone from flesh, 1 g., anhydrous dextrose, 1 g., glycerin, 2 g., water 100 g., tartaric acid, 0.07 per cent	No development.	No development.	No development
Oils Otto Rahn's solution (phosphate of potash, 0.5 g. sulphate of mag- nesium, 0.1 g., phos- phate of ammonia, 0.5 g., ferric chloride, trace, hydrochloric acid, trace, distilled water, 100 g.) plus 2 per cent olive oil Cohn's solution plus 2 per cent olive oil	In 20 to 30 days increase of free fatty acids mini- mum, showing extremely weak lipolytic action. Drops of oil in the cultures, consisting of a very delicate external wrinkled pellicle within which were seen vacuoles, more or less large, filled with bacteria	Faint clouding in 4 days. At 18 days, still a faint clouding, oil splitting up into globules, slight lipolytic action	Same as Barf <i>susutano</i> (Portofino)
Peptone water, precipi- tated carbonate of lime, and 2 per cent olive oil	do.-----	Good growth in 4 days, white layer at surface, flocks throughout In 13 days crystals hung from underside of pellicle In 18 days growth still heaviest at top No splitting of oil Growth in 4 days No splitting of olive oil even at 21 days	Do Do Do Do
Resistance to copper lithi- um, or nickel in Cohn's solution plus dextrose and Copper sulphate-- 1 300,000----- 1 200,000----- 1 100,000-----	Growth occurred. No growth. do.	Medium clouded in 3 days. Many crystals at surface in 9 days. do.	Do No growth Do
Nickel sulphate 10,000-----	Growth occurred	No growth in 2 days, fair amount of growth in 8 days	No growth in 8 days, growth in 18 days, partial rim, no pellicle
Lithium sulphate 1 1,000-----	do.	Slight growth in 3 days, good growth in 9 days.	Slight growth in 3 days, good growth in 9 days

TABLE 2—Comparison of cultural and physiological tests of the olive-tubercle organism (*Portofino strain*) and of the ash-ranker organism with *Petri's tests of has olive-tubercle strain*—Continued

Medium	Olive organism (Petri's, Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Uchinsky's solution, 100 g, ylose, 3 g. For production of gas	Cultures in fermentation tubes. Slight production of gas, fluid became acid	No gas production.	No gas production
Witte's peptone, 1 g, indigo carmine (0.5 per cent solution), 2 g, distilled water, 100 g. Test 1.....	Bottom of tube discolored after 48 hours, after 20 days colored ring remained at the surface	Moderate growth. Color changed uniformly to a darker shade of blue-green, darker than the ash organism	Moderate growth. No discoloring in rings. Medium deeper blue-green
Test 2.....	Color restored, but less intense, by current of air passed into fluid	Moderate amount of growth in 4 days. Color unchanged. Controls faded. In 6 to 21 days, color dark cinnamon green. Controls faded out entirely	Same as <i>Bact. savastanoi</i> (Portofino) except (at 6 to 21 days) color a tint deeper with a trifle more blue but still dark cinnamon green
Phosphate of potash, 0.02 g, agar, 1.5 g, mannite, 2 g, distilled water, 100 g	Rapid development, gradually slowing down, colony small. Bacteria very motile. Some motionless, united in chains, parallel to each other in sinuous bundles. After 15 days vacuolated individuals, were observed (degenerated forms)	Pin-point colonies up in 4 days. In 6 days pearl-like, translucent, white, higher in center, 1 to 1.5 mm in diameter. Fish-scale-like internal markings. In 8 days colonies 2.5 mm in diameter, raised center indented. In 10 days bacteria motile, not in chains, no vacuolated forms seen	Like <i>Bact. savastanoi</i> (Portofino) until 6th day. In 10 days largest colonies 1.5 mm in diameter, not so thick in center as <i>Bact. savastanoi</i> (Portofino). Bacteria motile, not in chains, no vacuolated forms seen
Winogradski's solution, 100 g, with Dextrose, 3 g.....	Moderate growth. Development stopped after 15 days. No butyric fermentation. Bacteria transferred to other substrata multiply actively. Less development than in Winogradski's solution with dextrose. Scarcely appreciable development.	Test 1. No growth. Test 2. Mere trace of growth even after 4 weeks.	Test 1. No growth. Test 2. Clouding in 3 days, heavy growth and pellicle in 2 weeks
Calcium propionate, 0.5 per cent Mannite, 3 per cent.....	do	Test 1. No growth. Test 2. No growth. Test 1. No growth. Test 2. No growth in 6 days, trace in 9 days, same at 4 weeks. Test 1. No growth. Test 2. No growth in 5 days, mere trace in 12 days, no heavier in 4 weeks. Test 1. No growth. Test 2. No growth.	Test 1. No growth. Test 2. No growth. Test 1. No growth. Test 2. No growth in 6 days, a fair amount of clouding with light pellicle in 9 days, very little heavier in 4 weeks. Test 1. Faint growth in all cultures. Test 2. Faint white growth in 5 days, same in 12 days, no further change in 4 weeks. Test 1. No growth. Test 2. Slight growth in 4 weeks.
Saccharose, 3 per cent.....	do		
Arabinose, 3 per cent.....	Good development, equaling that in Winogradski's solution with dextrose		

The detailed cultural work outlined in Tables 1 and 2 was carried out in order to ascertain whether the differences between the ash-canker and the olive-tubercle organisms would justify the separation of the ash organism as a distinct species

Although the olive-tubercle and the ash-canker organisms did not cross-inoculate in any of the tests and differed in some of their cultural characters, they were alike in the majority of the tests, morphologically they are essentially alike. The difference may be accounted for by the influence of the host on its particular parasite, an influence which has persisted to a marked degree during the 17 years in which the two organisms have been subcultured. The ash-canker organism, therefore, has not been made a separate species, but is here regarded as a variety of the olive-tubercle organism, *Bacterium savastanoi*, for which the varietal name *fraxini* is suggested.

The differences between Petri's olive-tubercle organism and the one used for this work (Portofino isolation) as indicated in Tables 1 and 2 are probably due to the differences in strains of the same organism. It may be noted that Petri's results correspond sometimes to those obtained with the olive-tubercle (Portofino) organism, sometimes to those obtained with the ash-canker organism, and sometimes to neither. Occasionally it happens that all three organisms give similar results. It is known that various bacteria exist in a variety of strains, and undoubtedly the olive-tubercle organism is no exception.

#### THE NEW VARIETY AND THE TYPE SPECIES COMPARED

##### *Bacterium savastanoi fraxini*, n. var.

The variety *fraxini* differs from *Bacterium savastanoi* mainly in specific host reactions, for the two organisms are only slightly separable by cultural or morphological characters. Table 3 shows the important differences between the two organisms.

TABLE 3.—Comparison of *Bacterium savastanoi fraxini*, n. var., with *Bact. savastanoi*.

Item	<i>Bact. savastanoi fraxini</i>	<i>Bact. savastanoi</i>
Pathogenicity.....	Produces cankers on ash trees, is not infectious to olive trees	Produces tubercles on olive trees, is not infectious to ash trees
Temperature range.....	5° to 32° C.	12° to 35° C.
pH range.....	5.6 to 8.5	6.5 to 9+
Cohn's solution.....	A green color develops in old flask cultures	No green color in old flask cultures.
Size of organisms from cultures of same age and stained the same	1.2μ to 3.3μ×0.4μ to 0.8μ	1.2μ to 1.5μ×0.4μ to 0.5μ

#### TREATMENT OF THE DISEASE

The organism causing canker on ash, like the olive-tubercle organism, is a wound parasite, and the treatment recommended for olive trees affected with tubercles may be suggested for the ash trees affected with cankers, namely, skillful pruning. As is usually recommended in treatment of this kind, the cut surfaces should be disinfected, as well as the pruning knives each time they are used.

Possibly a germicidal spray or germicidal paint might be used effectively at the earliest stage of the disease, when the split in the bark is very small and fairly regular, but in the later stage, when the

TABLE 2.—*Comparison of cultural and physiological tests of the olive-tubercle organism (Portofino strain) and of the osh-canker organism with Petri's tests of his olive-tubercle strain—Continued*

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Resistance to the following percentages of tartaric acid in Witte's peptone, 1 g, anhydrous dextrose, 1 g, glycerin, 2 g, water, 100 g—			
0.1 per cent.	No growth.	No growth.	No growth
0.07 per cent.	Weak growth.	do.	Do
0.03 per cent.	Elongation of bacteria.	(Good growth, no pellicle. In 6 days, many cross (X) forms. A few elongated forms and a few chains, but bacteria were mostly short	Heavy growth, thin white pellicle. In 6 days, all short forms no elongated ones nor X forms. Pellicle composed largely of dead bacteria. Pellicle composed largely of dead bacteria. Short forms prevalent, clumps present and a few Y's, bacteria motile
		In 15 days, short forms prevalent, clumps present and a few Y's, bacteria motile	In 6 days, short forms prevalent, clumps present and a few Y's, bacteria motile
		(Good growth, no pellicle. In 6 days short chains, some elongated forms and clumps	Good growth, incomplete pellicle and rim
	do	In 15 days small clumps, short chains, and a few Y's, bacteria motile	In 6 days, no clumps of bacteria nor elongated forms
0.005 per cent.			In 15 days small clumps, short chains, and a few Y's, bacteria motile

# NITROGEN-BALANCE STUDIES WITH VARIOUS FISH MEALS<sup>1</sup>

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## INTRODUCTION

In an earlier report<sup>2</sup> of fish-meal studies by the agricultural experiment station at Cornell University, it was shown that, as measured by growth experiments with rats, the protein of a vacuum-dried white fish meal was superior to that of a steam-dried menhaden meal. Both products proved superior to a flame-dried menhaden meal. It seemed probable that the differences in heat treatment were responsible, at least in part, for the differences in protein efficiency. It was recognized that the effect of higher temperature might show itself either in a lower digestibility or in a lower utilization of the absorbed nitrogen. It was therefore deemed desirable to extend the previous studies by the use of methods which would give more specific information regarding the differences in protein efficiency. The present paper reports the results of nitrogen-balance studies in which the three products previously mentioned were fed to rats and pigs.

The vacuum-dried white fish meal was a commercial product and consisted of the heads, tails, fins, and adhering flesh obtained as a by-product of the fillet industry and dried in vacuum under 105° F. It did not contain the entrails. The flame-dried menhaden meal was also a commercial product containing the whole fish, less most of the oil. This product was dried by direct heat at temperatures of approximately 500° F. The steam-dried menhaden meal was a product experimentally produced by the Bureau of Fisheries<sup>3</sup>. As compared with the flame-dried meal, it was undoubtedly more carefully handled throughout the manufacturing process as well as dried at a much lower temperature. The analyses of the samples used in the present study are shown in Table 1. Two experiments with growing rats and one with growing pigs were carried out, in which Mitchell's method<sup>4</sup> of computing the biological values from the nitrogen-balance data was used.

TABLE 1—Percentage analyses of the fish meals studied

Fish meal	Moisture	Ash	Crude protein	Ether extract
Flame-dried menhaden.....	5 12	21 13	57 95	6 13
Steam-dried menhaden.....	7 00	16 50	59 68	11 83
Vacuum-dried white.....	9 90	19 25	62 02	2 63

<sup>1</sup> Received for publication Dec 4, 1931, issued May, 1932. This paper presents a part of a thesis presented to the graduate school of Cornell University in partial fulfillment of the requirements for the degree of doctor of philosophy.

<sup>2</sup> MAYNARD, L. A., BENDER, R. C., and McCAY, C. M. VITAMIN A AND PROTEIN STUDIES WITH VARIOUS FISH MEALS. Jour Agr Research 44 591-603, illus 1932

<sup>3</sup> HARRISON, R. W. THE MENHADEN INDUSTRY. U. S. Dept Com, Bur Fisheries Invest Rpt 1, 113 p, illus 1931

<sup>4</sup> MITCHELL, H. H. A METHOD OF DETERMINING THE BIOLOGICAL VALUE OF PROTEIN. Jour Biol Chem. 58 873-903 1924

bark has become corrugated and corky, such treatment would result only in destroying the surface bacteria.

Since a slime containing bacteria oozes from the cankers when they are moist, it is quite possible that the disease may be spread from one branch to another by rain, the organism entering the tissues through very small wounds

#### SUMMARY

A bacterial organism isolated from a canker disease of the European ash tree is likewise infectious to the American ash, causing the same type of lesion. The disease has not been reported on the American ash in this country. The cankers of the European ash vary in size from small cracks with thickened margins to irregular fluted out-growths several inches in length and width, with cavities extending into the wood. They increase in size and number from year to year on both trunk and branches.

The organism isolated from the ash cankers is similar to *Bacterium savastanoi* E. F. Smith, which produces tubercles on olive trees in California, Italy, and other countries. Although the organisms do not cross-inoculate—the ash proving noninfectious to the olive and the olive noninfectious to the ash—both cultural and morphological tests show that they are essentially alike. The ash-canker organism is therefore regarded as a variety of the olive-tubercle organism, *Bact. savastanoi*, and the name *Bact. savastanoi* variety *fraxini* is suggested.

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TABLE 2.—Data obtained with male and female rats in first experiment to determine the protein efficiency of fish meals

## NITROGEN-FREE DIET

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Average daily urinary N	Average daily endogenous N	Average daily N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	Grams	Grams	Mili-grams	Mili-grams	Mili-grams	Mili-grams	Mili-grams	Mili-grams	Per cent	Per cent
Number 1♂	108	99	4.61	12.4	2.69	32.1	31.0	6.31	0	—	—
Number 2♂	130	125	6.78	17.4	2.57	61.4	16.7	21.3	—	—	—
Number 3♂	88	79	2.80	6.9	2.46	25.6	20.6	6.25	—	—	—
Number 4♂	129	119	6.86	17.7	2.58	26.7	27.7	26.7	—	—	—
Number 5♂	102	91	3.77	11.5	3.05	34.0	29.4	—	—	—	—
Number 6♂	116	115	6.24	12.5	2.00	—	—	—	—	—	—

## FLAME-DRIED MENHADEN FISH MEAL DIET

Number 1♂	101	101	4.80	78.2	33.6	55.2	28.5	9.7	45.5	56	82
Number 2♂	132	129	4.92	78.1	21.6	54.5	22.3	16.3	38.2	54	70
Number 3♂	108	104	5.81	87.5	22.0	65.5	34.0	10.0	55.5	59	85
Number 4♂	127	120	4.13	65.6	14.2	45.5	47.9	20.2	25.3	54	56
Number 5♂	125	127	5.73	91.0	18.0	73.0	40.4	24.1	50.7	64	69
Number 6♂	131	131	5.63	87.8	22.4	65.4	53.1	25.1	40.3	61	62

## STEAM-DRIED MENHADEN FISH MEAL DIET

Number 1♂	113	112	4.56	70.0	21.2	60.5	35.7	7.1	53.4	70	84
Number 2♂	130	129	4.83	74.1	23.6	63.2	38.5	15.7	47.5	68	75
Number 3♂	109	109	5.07	77.8	26.6	64.2	40.3	21.1	43.5	66	67
Number 4♂	130	121	4.41	67.7	20.8	57.8	65.4	39.2	18.6	69	32
Number 5♂	106	100	5.03	77.2	24.3	67.6	30.7	5.9	61.7	69	91
Number 6♂	117	120	4.91	75.4	20.6	65.0	34.8	3.2	61.8	73	95

## VACUUM-DRIED WHITE FISH MEAL DIET

Number 1♂	127	138	6.84	112.8	23.8	106.2	42.9	12.8	93.4	79	88
Number 2♂	135	131	4.66	76.7	19.2	69.8	55.6	31.7	38.1	75	55
Number 3♂	97	102	4.53	74.5	16.0	69.9	33.6	9.3	60.6	79	87
Number 4♂	126	139	6.04	99.4	19.8	94.9	39.2	11.8	83.1	80	88
Number 5♂	117	123	6.44	105.9	27.2	96.6	47.4	11.0	85.0	74	89
Number 6♂	117	134	6.03	99.2	21.0	91.1	32.7	12.6	78.5	79	86

\* Per gram of food

\* This value was estimated as not enough food was eaten during this period to supply the energy requirements of the rat

## RAT EXPERIMENTS

In each experiment six rats were used. In the first, three females were included, but in the second, males only were employed. After from five to seven days on a nitrogen-free ration consisting of 15 per cent of butterfat, 12 per cent of lard, 2 per cent of cod-liver oil, 4 per cent of salt mixture (Osborne and Mendel), and 67 per cent of starch, the urine and feces were collected for a 7-day period. Then followed three 10-day experimental periods in which the three fish meals were fed at such levels that their proteins formed 10 per cent of the ration, the calorific and mineral content being kept the same. Vitamin B concentrate (Osborne and Wakeman) was included in the ration in the first experiment at a 0.15 per cent level and in the second experiment at a 1.6 per cent level.<sup>5</sup> The collections of excreta were made the last seven days of each 10-day period. The experiments were each ended as they were begun, with a nitrogen-free period. The diets and all the excreta were analyzed for total nitrogen by the Kjeldahl method.

The data obtained in the first and second rat experiments are summarized in Tables 2 and 3 respectively. The two values, the digestion coefficient and the biological value, express as percentages the part of the food nitrogen which is realized by the body at each of the two points in the organism where waste occurs. The digestion coefficient represents the percentage of crude protein ( $N \times 6.25$ ) digested and thus saved for body use. The biological value expresses the percentage of absorbed protein which has been used in anabolism.

By averaging all the protein-digestion coefficients obtained for a given product, as listed in Tables 2 and 3, the following mean values are obtained: Flame-dried menhaden meal,  $62.2 \pm 1.15$ ; steam-dried menhaden meal,  $73.2 \pm 0.96$ , vacuum-dried white meal,  $80.7 \pm 0.73$ . Thus, the vacuum-dried white fish meal showed an advantage of  $7.5 \pm 1.20$  over steam-dried menhaden, the difference being 6.2 times its probable error. The digestibility of the steam-dried menhaden showed an advantage of  $11.0 \pm 1.50$  over the flame-dried menhaden fish meal, the difference being 7.3 times its probable error.

By averaging the biological values in Tables 2 and 3 the following mean values are obtained: Flame-dried menhaden meal,  $71.7 \pm 1.54$ , steam-dried menhaden meal,  $77.4 \pm 3.15$ , vacuum-dried white meal,  $83.4 \pm 1.83$ . Analysis of these averages of values from both Table 2 and Table 3 reveals the fact that the difference of  $5.7 \pm 3.51$  between the biological values of flame-dried menhaden and steam-dried menhaden is not significant. The same is true of the difference of  $6.0 \pm 3.64$  between steam-dried menhaden and vacuum-dehydrated white fish meal. The large probable errors which keep these differences from being considered significant are due primarily to the high degree of variation shown by certain of the values obtained in the first experiment, which will be discussed later. The difference of  $11.7 \pm 2.39$  between flame-dried menhaden and vacuum-dried white fish meal is clearly significant, the difference being 4.9 times its probable error. The white fish meal is shown to be distinctly more efficiently used in anabolism than is the flame-dried menhaden. This is borne out by the swine experiment, which is discussed later.

<sup>5</sup> In the first experiment, a minimum amount of vitamin B concentrate was used to avoid too great an addition of nitrogen from a source other than fish meal. The larger amount used in the second experiment was based upon a test of the potency of the product used.

## FLAME-DRIED MENHADEN FISH MEAL DIET

Number 1.....	70	84	5 28	87 9	24 9	10 3	14 6	73 3	38 2	20 6	17 6	55 7	72	76
Number 2.....	85	92	6 01	100 0	35 7	12 7	23 0	77 0	46 5	24 8	21 7	55 3	64	72
Number 3.....	122	126	6 40	106 5	44 3	14 9	29 4	77 1	53 2	20 0	24 2	52 9	58	69
Number 4.....	161	165	8 06	134 1	45 9	16 0	29 9	104 2	69 4	27 0	32 4	71 8	66	69
Number 5.....	112	119	5 06	84 2	27 1	9 3	17 8	66 4	49 7	26 9	16 7	49 7	68	75
Number 6.....	122	140	9 64	160 4	48 1	20 5	27 6	132 8	71 5	37 7	33 8	99 0	70	75

## STEAM-DRIED MENHADEN FISH MEAL DIET

Number 1.....	116	129	7 84	138 6	28 3	16 9	11 4	127 2	54 8	31 0	23 8	103 4	80	81
Number 2.....	123	134	7 93	140 2	30 1	19 5	10 6	129 6	61 1	32 3	28 8	100 8	79	78
Number 3.....	112	122	6 36	112 4	32 8	14 1	18 7	93 7	47 2	30 9	16 3	77 4	71	83
Number 4.....	132	158	9 54	168 7	37 6	18 7	38 9	149 8	58 8	34 2	24 6	123 2	78	84
Number 5.....	102	115	6 38	97 4	22 4	11 9	10 5	86 9	43 9	26 8	17 1	69 8	77	80
Number 6.....	95	113	9 17	139 9	31 1	17 6	13 5	126 4	62 6	31 4	31 2	95 2	76	75

## VACUUM-DRIED WHITE FISH MEAL DIET

Number 1.....	94	114	6 96	114 0	19 8	14 3	5 5	109 1	42 8	27 0	15 8	93 3	83	86
Number 2.....	102	123	7 08	126 5	23 9	17 5	6 4	120 1	46 4	29 8	16 6	103 5	81	86
Number 3.....	92	111	7 28	119 9	18 2	15 4	2 8	117 1	42 0	27 8	12 8	104 3	85	89
Number 4.....	101	125	7 98	131 4	20 8	15 5	5 3	125 1	46 0	29 8	18 2	107 9	84	86
Number 5.....	122	130	5 87	88 4	12 6	9 8	2 8	85 0	44 4	27 5	16 0	68 7	86	80
Number 6.....	147	158	9 05	149 1	25 1	21 2	3 9	145 2	69 1	41 9	27 2	118 0	83	81

## NITROGEN-FREE DIET

Number 1.....	121	107	6 17	-----	13 8	a 2 24	-----	-----	28 1	b 24 6	-----	-----	-----	-----
Number 2.....	127	105	5 84	-----	15 4	a 2 64	-----	-----	27 4	b 23 6	-----	-----	-----	-----
Number 3.....	119	110	3 77	-----	9 2	a 2 44	-----	-----	23 4	b 20 4	-----	-----	-----	-----
Number 4.....	155	149	6 80	-----	13 7	a 2 01	-----	-----	33 1	b 21 8	-----	-----	-----	-----
Number 5.....	125	116	4 09	-----	7 4	a 1 81	-----	-----	24 6	b 20 4	-----	-----	-----	-----
Number 6.....	147	134	7 05	-----	18 0	a 2 55	-----	-----	36 8	b 26 2	-----	-----	-----	-----

a Per gram of food

b Per 100 grams live weight

TABLE 2—Data obtained with male and female rats in first experiment to determine the protein efficiency of fish meals—Continued

## NITROGEN-FREE DIET

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	Grams	Grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Per cent	Per cent
Number 1♂	135	125	5.74	—	14.1	±2.46	—	—	25.9	619.9	—	—	—	—
Number 2♂	129	125	5.66	—	15.1	±2.67	—	—	23.5	618.5	—	—	—	—
Number 3♂	106	99	4.13	—	11.2	±2.71	—	—	23.1	622.5	—	—	—	—
Number 4♂	124	114	6.43	—	15.6	±2.43	—	—	25.0	621.0	—	—	—	—
Number 5♂	127	120	5.59	—	14.1	±2.52	—	—	20.1	616.3	—	—	—	—
Number 6♂	131	123	5.36	—	12.2	±2.28	—	—	23.8	618.7	—	—	—	—

TABLE 3—Data obtained with male rats in second experiment to determine the protein efficiency of fish meals

## NITROGEN-FREE DIET

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	Grams	Grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Milli-grams	Per cent	Per cent
Number 1	88	74	4.24	—	7.7	±1.87	—	—	8	27.5	—	—	—	—
Number 2	82	77	4.22	—	8.3	±1.86	—	—	24.6	629.3	—	—	—	—
Number 3	91	77	3.58	—	8.2	±2.01	—	—	27.2	632.4	—	—	—	—
Number 4	107	91	4.84	—	9.7	±2.92	—	—	25.2	626.4	—	—	—	—
Number 5	108	90	3.88	—	7.3	±1.88	—	—	25.9	626.2	—	—	—	—
Number 6	95	77	4.27	—	7.3	±1.71	—	—	27.1	631.5	—	—	—	—

was used from five days prior to the first nitrogen-free collection period through this period. It was also used in the final nitrogen-free period. Pig No. 1 was inclined at first to be somewhat irregular in fecal excretion. During the last day of what was intended to be the first 7-day nitrogen-free collection period, he excreted no feces at all. This day was discarded and only six days counted for this first period.

The plan followed in the swine experiment was as follows.

Preliminary period on nitrogen-free ration.....	19 days.
Collection period on nitrogen-free ration.....	7 days
Preliminary period on fish-meal rations.....	5 days
Collection period on fish-meal rations.....	7 days
Collection period on fish-meal rations.....	7 days
Preliminary period on fish-meal rations.....	5 days
Collection period on fish-meal rations.....	7 days
Collection period on fish-meal rations.....	7 days
Preliminary period on nitrogen-free ration.....	5 days
Collection period on nitrogen-free ration.....	7 days

During the first preliminary period on the fish-meal rations, and the two collection periods following, pig No. 1 received the flame-dried menhaden fish meal ration, and pig No. 2 received the vacuum-dried white fish meal ration. During the second preliminary period in which the fish-meal rations were fed, the rations were reversed so that pig No. 1 received the white meal and pig No. 2 the menhaden meal.

As in the rat experiments, in the swine experiment the fish meals were mixed with the nitrogen-free ration at such levels that their proteins formed 10 per cent of the ration. The fish meal replaced part of the starch and minerals of the nitrogen-free ration. Only enough mineral mixture was added to make the total mineral content of each ration 4 per cent. The rations used are shown in Table 4. Cod-liver oil was fed at the rate of 16 g per pig per day.

The pigs were fed twice a day. Throughout the experiment the rations fed each day were entirely consumed; therefore no refused feed had to be accounted for. Each pig vomited slightly once during the last fish-meal collection period, but the amount involved less than 1 gram of dry matter. It was concluded that the amount of nitrogen lost was insignificant as compared with the weekly nitrogen intake.

TABLE 4 — *Composition of the fish meal rations used in the swine feeding experiment*

Constituent	Composi- tion of the flame-dried menhaden fish meal ration	Composi- tion of the vacuum- dried white fish meal ration
	<i>Parts</i>	<i>Parts</i>
Fish meal.....	15.88	14.83
Starch.....	73.62	74.17
Mineral mixture.....	50	1.00
Cellophane.....	8.00	8.00
Yeast.....	2.00	2.00

The data from both rat experiments indicate the probability that the position of the biological value of steam-dried menhaden is intermediate between the biological values of the other two fish meals studied. If the data of the second rat experiment (Table 3) are taken separately, this is more definitely shown. In drawing conclusions regarding the biological values, the author believes that the results of the second rat experiment are much more accurate than those of the first. The potency of the vitamin B concentrate was tested prior to the second experiment, and as a result a much larger quantity was used than in the first experiment. Larger food consumption and more nearly normal growth resulted. Also, as the most erratic biological values in the first experiment occurred with female rats, only males were used in the second experiment. Further, it is likely that during the second experiment the author was more adept in all the techniques involved. There were no widely diverging values in the second experiment. This resulted in much smaller probable errors, even though the average values of the second experiment included only half as many observations as were included in the two experiments.

The average biological values obtained in the second rat experiment are  $72.7 \pm 0.86$ ,  $80.2 \pm 0.91$ , and  $84.7 \pm 0.95$  for the proteins of flame-dried menhaden, steam-dried menhaden, and vacuum-dried white meal, respectively. The white fish meal shows an advantage over steam-dried menhaden of  $4.5 \pm 1.32$ , the difference being 3.4 times its probable error. The steam-dried menhaden shows an advantage over the flame-dried menhaden of  $7.5 \pm 1.25$ , the difference being 6.1 times its probable error. Thus, on the basis of the second rat experiment taken alone, it is not necessary to resort to inference to decide that the position of the steam-dried product is intermediate between the other two fish meals with respect to the utilization of its protein after absorption. The second experiment when taken alone also shows the greater biological value of the white fish meal as compared with the flame-dried menhaden by the more significant difference of  $12.0 \pm 1.28$ , the difference being 9.4 times its probable error.

#### THE SWINE EXPERIMENT

As fish meal is primarily a swine and poultry feed, a further comparison in which only the two commercial meals, flame-dried menhaden and vacuum-dried white meal, were used was made with swine. Two young Berkshire barrows from the same litter, weighing 37.3 and 31.8 kilograms, respectively, were used. Essentially the same technique was followed as with rats. The pigs required a much longer nitrogen-free feeding period before the endogenous level of nitrogen excretion was reached. Daily samples of urine were taken after the second week until what appeared to be a constant and sufficiently low level was reached.

After several attempts, a nitrogen-free ration was mixed with which it was possible to secure more or less uniform excretion of feces. Before collections were made, the composition of the nitrogen-free ration was established at 8 per cent of cellophane, 4 per cent of a mineral mixture, and 88 per cent of starch. The mineral mixture consisted of 40 parts of calcium phosphate, 40 parts of ground limestone, 20 parts of sodium chloride, and 1 part of ferrous sulphate. This ration

The results of the swine experiment are shown in Table 5. This limited experiment, in which only two pigs were used, is in reality of greater significance than would be the case if the data were not in agreement with those from the 12 rats. The results with even two pigs are significant under such circumstances. Attention is called to the fact that the rat rations and the pig rations were not entirely comparable. The fish-meal proteins were present in each at a 10 per cent level by analysis. However, because of the high fat content of the rat rations the percentage of protein calories was only 8 per cent, whereas with the swine rations the cellophane and mineral content caused the percentage of protein calories to be 12 per cent, if it is assumed that no calories are derived from the cellophane.

An inspection of the digestion coefficients and biological values in Table 5 shows clearly that the pig experiment supports the work with rats in showing the superiority of the proteins of the white fish meal. Since the data were furnished by only two animals, the values were not averaged nor treated statistically. It is evident however, that the protein of the vacuum-dried white fish meal was decidedly more efficient than the protein of the flame-dried menhaden fish meal, with each pig, in both digestion and anabolism. It will be noticed that throughout the experiment pig No. 2 tended to utilize protein more efficiently than pig No. 1.

The experiments described in this paper confirm those of Maynard, Bender, and McCay<sup>6</sup> in showing that the products studied rank in the following order as regards protein efficiency: Vacuum-dried white fish meal, steam-dried menhaden meal, flame-dried menhaden meal. In addition, they supply more specific and quantitative information in showing that the differences result in part from differences in digestibility and in part from differences in the utilization of the absorbed nitrogen. Further, they indicate that the results obtained with the rats hold also for growing pigs and thus furnish further evidence as to the usefulness of the rat for pilot experiments with swine rations. In view of the fact that fish meals are used primarily as protein supplements in both swine and poultry rations, the results obtained with the two commercial meals are of obvious practical importance. They suggest the desirability of further studies, not only of various products now sold for stock feeding, but also of the conditions both as to raw material and processing which provide proteins of high biological value.

#### SUMMARY

In two nitrogen-balance studies involving a total of 12 growing rats the products studied were found to rank in the following order as regards the digestibility of their proteins: Vacuum-dried white fish meal, steam-dried menhaden meal, flame-dried menhaden meal. A comparison of the white meal and the flame-dried menhaden by the same procedure with two growing pigs produced results similar to those obtained with the rats.

In both rat experiments and in the swine experiment the vacuum-dried white meal proved significantly superior to the flame-dried

<sup>6</sup> MAYNARD, L. A., BENDER, R. C., and MCCAY, C. M. *Op. cit.*

TABLE 5.—Data obtained with swine in the experiment to determine the protein efficiency of fish meals

NITROGEN-FREE RATION

Pig	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in urine	Average daily food N unabsorbed	Digestion coefficient	Biological
No 1 <sup>a</sup>	Kgm	Kgm	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Grams	Per cent	Per cent
No 2	47 3 31 8	30 9 31 3	700 600	0 68 0 54	6 0 97 6 0 90	2 12	1 98	2 12	2 00	2 00	2 00	6 51 6 10 7 27 7 05	73 72 74 75	69 69 74 74

FLAME-DRIED MENHADEN FISH MEAL RATION

No 1	38 3	39 0	750	11 99	3 18	69	2 49	9 50	4 94	1 05	2 90	6 51	73	69
No 1	39 6	40 2	750	11 99	3 36	68	2 38	9 31	5 93	1 92	3 21	6 10	72	69
No 2	35 6	36 9	750	11 99	3 06	83	2 23	9 70	4 80	2 11	2 40	7 27	74	74
No 2	36 9	37 7	750	11 99	2 98	80	2 12	9 87	4 91	2 00	2 82	7 05	75	74

VACUUM-DRIED WHITE FISH MEAL RATION

No 1	42 3	44 3	870	13 83	1 72	72	1 00	12 83	4 84	1 94	2 90	9 93	83	77
No 1	44 3	45 7	850	13 83	1 31	71	63	13 20	4 76	1 93	2 83	10 37	90	79
No 2	32 7	33 7	650	10 68	1 03	64	39	10 19	3 92	2 12	1 80	8 38	90	82
No 2	33 7	35 1	650	10 68	1 01	66	38	10 20	3 74	2 12	1 62	8 58	90	84

NITROGEN-FREE RATION

No 1	45 4	45 6	775	61	6 78	1 81	0 040	1 96	0 052	0 052	0 052	0 052	0 052	0 052
No 2	37 4	37 6	675	83	6 122	1 96	0 052	1 96	0 052	0 052	0 052	0 052	0 052	0 052

<sup>a</sup> This is a 6-day collection period instead of a 7-day period

<sup>b</sup> Fecal nitrogen per kilogram of dry feed. These values were used in estimating the metabolic nitrogen in the feces in the fish-meal periods. The change in the ratio of metabolic nitrogen to dry matter consumed from the first to last periods was assumed to occur in a linear fashion.

<sup>c</sup> Urinary nitrogen per kilogram of body weight. These values were used in estimating the endogenous (body) nitrogen in the urine in the fish-meal periods, the same assumption of a linear variation from the first to last periods being made as in the case of the metabolic nitrogen in the feces.

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## A CYTOLOGICAL STUDY OF HETEROTHALLISM IN PUCCINIA TRITICINA<sup>1</sup>

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### INTRODUCTION

The announcement by Craigie in 1927 of heterothallism in the rusts has opened up a new field of research of both theoretic and practical interest. The first paper (?)<sup>3</sup> in which experimental proof was given of heterothallism in *Puccinia helianthi* Schw. was soon followed by a second paper (8, p 765), presenting similar proof for *P graminis* Pers. Here, too, evidence was given that the pycnia or spermogonia are not,

as many botanists have supposed, male conceptacles producing non-functional spermatia, but are active organs having a non-male function which they carry out through the agency of flies \* \* \* the mycelium, pycnia, and pycnospores of some of the pustules were (+) in sex, whereas the mycelium, pycnia, and pycnospores of other pustules were (-) in sex.

When a monosporidial pustule is kept isolated it produces pycniospores (haploid) but no aeciospores (diploid). But when the nectar (exudate containing the spores) of a (+) infection is transferred to the nectar of a (-) infection (or vice versa), aeciospores form. The pycniospores (spermatia) bring about the change from the haploid to the diploid phase.

In a third paper by Craigie (9) additional data are given in support of the above conclusions, and field observations are recorded which indicate that *Puccinia coronata* Cda, *P. pringsheimiana* Kleb, and *Gymnosporangium* sp. also are heterothallic.

While these studies established the fact of heterothallism in *Puccinia graminis*, nothing was yet known of the actual process taking place in the host plant. However, in 1929 Hanna (12) published a preliminary account of studies in this field. He found that in the pustule of monosporidial origin the mycelium and pycnia are uninucleate and that such an infection produces, near the lower epidermis of the leaf, sterile wefts, "which appear to be crescent-shaped in trans-

<sup>1</sup> Received for publication Nov 19, 1931, issued June, 1932. Cooperative investigations of the Agricultural Experiment Station, University of California, and the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U S Department of Agriculture.

<sup>2</sup> Acknowledgments are made to M A McCall and H B Humphrey, both of the Division of Cereal Crops and Diseases, for a careful reading of the manuscript, to H B Humphrey, Margaret Newton, of the Dominion Rust Research Laboratory, Winnipeg, Canada, C O Johnston, of the Division of Cereal Crops and Diseases, and E B Mains, formerly agent of that division, for spores of *Puccinia tritici*; to E B Mains for roots of *Thalictrum flavum*, and to members of the Divisions of Agronomy and Genetics of the University of California for courtesies extended during the investigation.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p 753.

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menhaden as regards the utilization of the absorbed nitrogen. In both rat experiments the white meal proved numerically superior in this respect to the steam-dried menhaden, and the latter numerically superior to the flame-dried product, but these differences were biometrically significant in the case of the second experiment only.

function as trichogynes are much branched and highly septate organs, having their terminus at the epidermis of the host leaf where they project through stomata or between epidermal cells and make contact with spermatia. Andrus found that the rust is heterothallic—an isolated infection remains haploid. When spermatia from one infection are transferred to a different infection and applied to the leaf surface, the nuclei of the spermatia enter the trichogynes and pass to their base, where they become associated with the native nuclei to form the beginning of the sporophyte generation. By proliferation of these cells, and sometimes by supplementary fusions, a sporogenous layer of binucleate cells is formed from which spring the spore chains of diploid cells. The Christman theory of fertilization (6) in rusts is held to be no longer tenable.

Much cytologic work on the aecial generation of rusts (1) was published prior to the discovery of heterothallism in this group. In such investigations stages of the isolated sterile infection, if accidentally encountered in the material studied, would either be incorporated with the rest as a part of the story of development of the fertile infection or be cast out as "pathologic." Moreover, previously accepted observations as to the mode of origin of the sporophyte generation in the aecium are now in need of a critical repetition in the light of recent discoveries. In order to further the knowledge of the microscopic details of heterothallism, a cytologic study of *Puccinia triticina* Eriks was undertaken by the writer.

#### MATERIALS AND METHODS

Spores of *Puccinia triticina* were obtained from E. B. Mains, H. B. Humphrey, Margaret Newton, and C. O. Johnston, and plants of *Thalictrum flavum* L. were obtained from E. B. Mains. Seed of another species of *Thalictrum* was purchased as *T. delavayi* Franch., but was probably *T. dipterocarpum* Franch. The plants proved susceptible to the rust.

The plants were grown in the greenhouse, and the experiments were carried out there. The straw bearing teliospores was placed in loose-mesh cloth bags. A part was kept in cold storage, and the rest was kept in the field, in a partly shaded spot on the ground. The latter method proved the more satisfactory.

In inoculating, watch crystals were filled with mud; bits of the rusted straw were soaked in rain water several hours and sprayed with an atomizer to stimulate germination, then pressed into the mud, with the spores exposed, and sprayed again. A bit of wet sphagnum was wrapped around the base of each plant. Tall glass tumblers with the bottoms removed and the sides lined with wet paper were placed over the plants and the mud-filled crystals used as lids. A layer of wet paper was folded down over the top and held in place with a rubber band. This placed the rusted straw directly above the leaves, and as the sporidia were formed and set free they fell on the leaves. The whole was placed under a greenhouse bench for 48 hours, then uncovered and replaced on the bench.

The inoculated plants were covered with tarlatan cages to exclude insects. These cages were effective in excluding flying insects, but did not keep out thrips or red spiders. Every effort was made to keep the plants free from these latter; but, despite precautions,

verse sections of the leaf" and which "are evidently haploid rudiments of aecial cups waiting to be stimulated into further developmental activity" He observed that pycniospores germinate, the largest germ tube seen being  $15\mu$  long, and that in the infection to which pycniospores of opposite sex have been transferred, the sporophyte generation appears and open aecia develop. He found that the first binucleate cells are formed in the base of the aecium by the fusion of cells in pairs in the manner described by Christman (6), the fusion cell becoming the basal cell of a spore chain.

In a paper covering the same ground the writer (1) agrees in the main with Hanna's observations but finds evidence that the sporophyte generation begins earlier. Mycelial cells with more than one nucleus have been found at the pycnium, in the mycelium, and in the young aecium before the sporogenous layer was differentiated. Fusion of cells at the base of the aecium to form 2-legged basal cells has not been seen.

The fact that in certain rusts the two cells that fuse to initiate the diploid generation come of different parents suggests the possibility that physiologic forms and varieties of those rusts may hybridize on the aecial host and that new physiologic forms may arise from such crosses. Experiments along this line are in progress in several laboratories and preliminary reports of three of these (16, 18, 20) have been published.

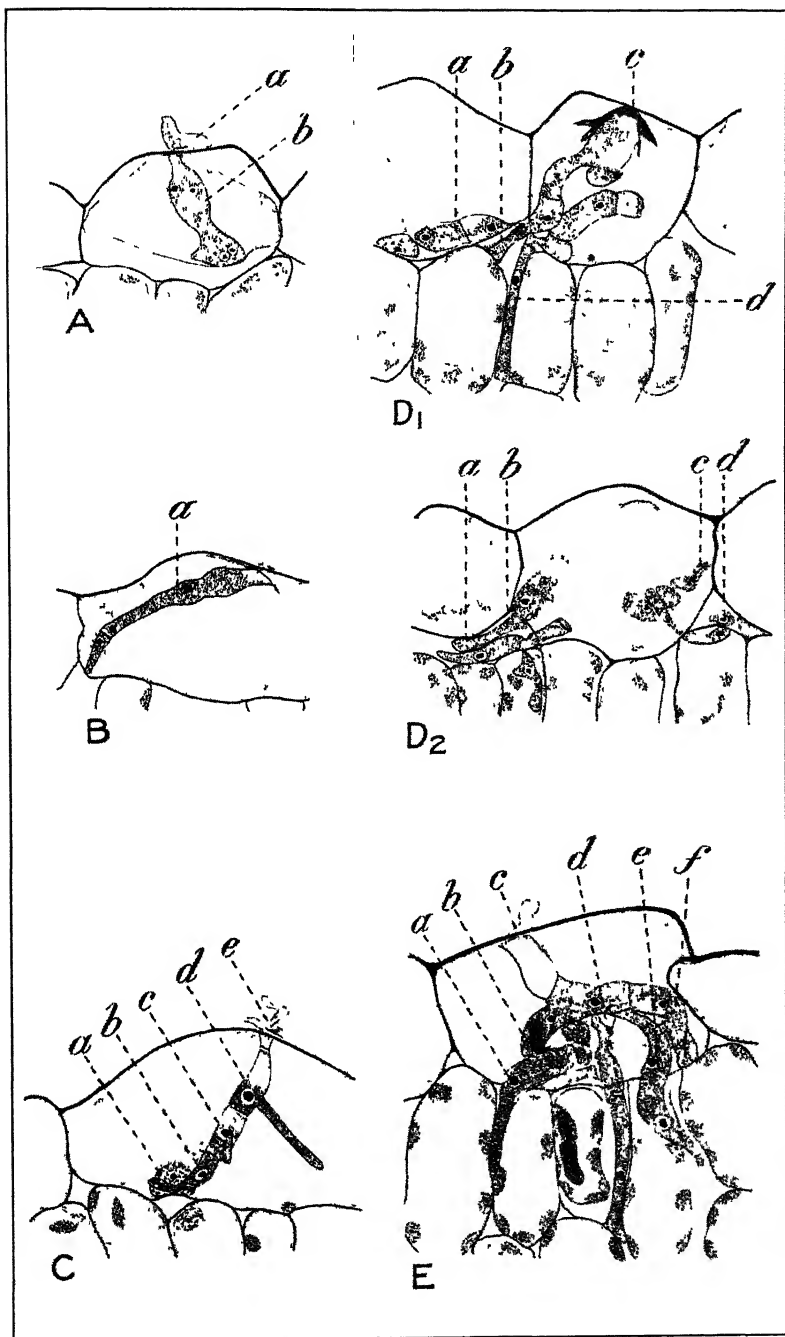
Waterhouse (20) in Australia crossed two physiologic forms of *Puccinia graminis tritici* Eriks and Henn. on the barberry and obtained from the cross two physiologic forms new to that continent.

According to Stakman, Levine, and Cotter (18), crosses between *Puccinia graminis tritici* and *P. graminis agrostidis* Eriks may result in a number of physiologic forms of *tritici*, some previously known and others new to science. From one cross eight physiologic forms were isolated, apparently from the spores of a single aecial cup. From crosses between *P. graminis tritici* and *P. graminis secalis* Eriks and Henn., the parental forms were recovered, together with other physiologic forms, some of which were new. In long-continued uredinial cultures of certain physiologic forms of *tritici* new physiologic forms have appeared by mutation.

According to Newton, Johnson, and Brown (16), the selfing of a physiologic form may result in several forms, some known previously and others new to science. Only one of the physiologic forms used in their experiment proved homozygous. They found that when 2 physiologic forms were crossed, in a few cases a parent form reappeared, but more commonly a different form appeared, and that when the mixed exudate of 8 physiologic forms was used in crosses, 17 forms appeared, of which 7 were new. Spores from each aecial cup in this experiment were cultured separately, in 95 per cent of the cases only 1 physiologic form was isolated from each cup.

Andrus (3) in a paper<sup>4</sup> presents an account of the gametophytic development and fertilization of *Uromyces appendiculatus* (Pers.) Fries and other rusts. Andrus found that in *U. appendiculatus* the sporidium gives rise to a haploid mycelium that bears spermogonia and trichogynes. He states that the gametophytic hyphae which

<sup>4</sup> A paper delivered before the mycological section of the Botanical Society of America in December, 1930, and made available to the present writer through the courtesy of its author. A report of this investigation (3) has appeared since the present paper was prepared.



occasional infections were fertilized through their agency. The infections were studied and greenhouse records of them were kept.

Material was fixed daily for the first three weeks and at longer intervals from then on until the infections were 6 weeks old. The fixing fluids used were chrom-acetic formalin and Flemming's medium and weak solutions. Of these, Flemming's weak solution was most trustworthy. The material, after remaining 36 to 48 hours in the fixing fluid, was washed, dehydrated, and embedded in 50° paraffin. The principal stains used were safranin and methylene blue

## INVESTIGATIONS

### ENTRANCE AND DEVELOPMENT OF MYCELIUM

After 24 hours in the inoculating chamber some of the teliospores had germinated, the promycelia had produced and freed sporidia which had fallen on the leaves, and some of the sporidia had then germinated and entered the epidermal cells of the leaves.

Plate 1, A, shows an epidermal cell with the young rust fungus from a 1-day infection<sup>5</sup>. The host cell is somewhat plasmolyzed. Flemming's medium solution, used in fixing this preparation, proved too strong for *Thalictrum* leaves. On the outer surface of the cell is the sporidium (*a*), which is only partly evacuated. Even in older preparations there is sometimes a remnant of the spore plasm left in the sporidium. The bulk of the sporidial content has entered to form the sacklike mass (*b*) within the epidermal cell. There are apparently six nuclei in this cell. A comparison with later stages (pl. 1, B and C) makes this seem doubtful, or at least exceptional. However, it would be expected that one or two nuclear divisions would take place before septation.

Plate 1, B, *a*, shows a newly septate primary hypha. It is slender, consisting of three uninucleate cells, and runs diagonally across the epidermal cell.

The primary hypha develops further, growing apically to form new cells, while the older cells become thicker and heavier and put forth branches. In Plate 1, C, drawn from a 2-day infection, is a 4-cell primary hypha (*a-d*) and the remnant of the original sporidium (*e*). The two older cells (*c* and *d*) have thickened and produced short branches. Their cytoplasm is vacuolate, much of it having flowed out into the branches, but the nuclei are still in the parent cells. The two younger cells (*a* and *b*) are still slender, dense, and unbranched.

In the later growth the primary hypha and its branches become heavier and somewhat tangled, and it is often difficult to trace their course. Plate 1, D<sub>1</sub> and D<sub>2</sub>, shows successive sections of a 3-day

<sup>5</sup> For the sake of uniformity and clearness, the drawings are oriented in the plates as the tissues are in the leaf, i. e., having the tissues nearest the upper surface of the leaf uppermost in the drawing.

### EXPLANATORY LEGEND FOR PLATE 1

- A—One-day infection. The sporidium (*a*) has germinated and entered the epidermal cell at *b* × 1,020.  
 B—Two-day infection. Primary hypha (*a*) composed of three uninucleate cells × 1,020.  
 C—Four-cell primary hypha (*a-d*) and remnant of sporidial wall (*e*) from 2-day infection. The older cells (*c* and *d*) are pushing out branches × 1,020.  
 D<sub>1</sub> and D<sub>2</sub>—Successive sections of a 3-day infection showing primary hypha (D<sub>1</sub>, *c*, and D<sub>2</sub>, *c*) and its branches (D<sub>1</sub>, *a*, *b*, and *d*, and D<sub>2</sub>, *a*, *b*, and *d*) × 1,020.  
 E—Three-day infection. Primary hypha entered at *c*, grew to *f*, and doubled back to *b*. Branches at *a*, *d*, and *e* × 1,020.

infection The primary hypha entered at  $D_1, c$ , swung to the left then curved back to the right, ending at  $D_2, c$ . Apparently, the terminal cell ( $D_2, c$ ) is unbranched. Not less than 6 branches (and probably more) have formed and grown out of the host cell, 2 into other cells (pl 1,  $D_1, a$ , and  $D_2, d$ ), and 4 into intercellular spaces (pl 1,  $D_1, b$  and  $d$ , and  $D_2, a$  and  $b$ ).

In contrast to *Puccinia graminis* (1), these branches from the primary hypha do not make their exit from the epidermal cell through a small pore in the host cell wall, but from a relatively large hole often equal in diameter to the hypha. This is evident in Plate 1,  $D_1$  and  $D_2$ , and shows still more clearly in Plate 1, E. Here (pl 1, E) the rust entered at  $c$ , and the primary hypha swung to the right at  $f$ , then doubled back to  $b$ . It consists of 6 cells, of which the 4 older have branched and are now more or less evacuated, while the 2 younger are still unbranched. Three of the large vigorous branches are undiminished in diameter at the point of exit from the epidermal cell (Pl. 1, E,  $a, d$ , and  $e$ .) These branches are making their way down between the palisade cells to the more open air spaces below.

Progress by the fourth day is shown in Plate 2, A. The primary hypha has entered at  $d$  and curved around to  $b$ . As before, the primary hypha itself does not leave the host cell, and its terminal cell ( $b$ ) is unbranched. Branches at  $a$  and  $c$  have grown down into the host tissues, forming the mycelium. Ordinarily a cell of the primary hypha gives rise to only one branch. The cell at  $d$ , however, has formed two branches, neither of which is effective.

Once the mycelium is established, little or no further development occurs at the point of entry, and degenerative changes of the primary hypha set in. Plate 2, B, drawn from a 7-day infection, shows the primary hypha ( $a$ ) breaking down, but a rich growth of branching hyphae radiates from it. This mycelium is haploid. The rapidly growing terminal cell of a hypha, however, may have two nuclei just before a septum divides it into two cells (Pl. 2, B,  $c$ ). Rarely, there may be more than two (Pl. 2, B,  $d$ ).

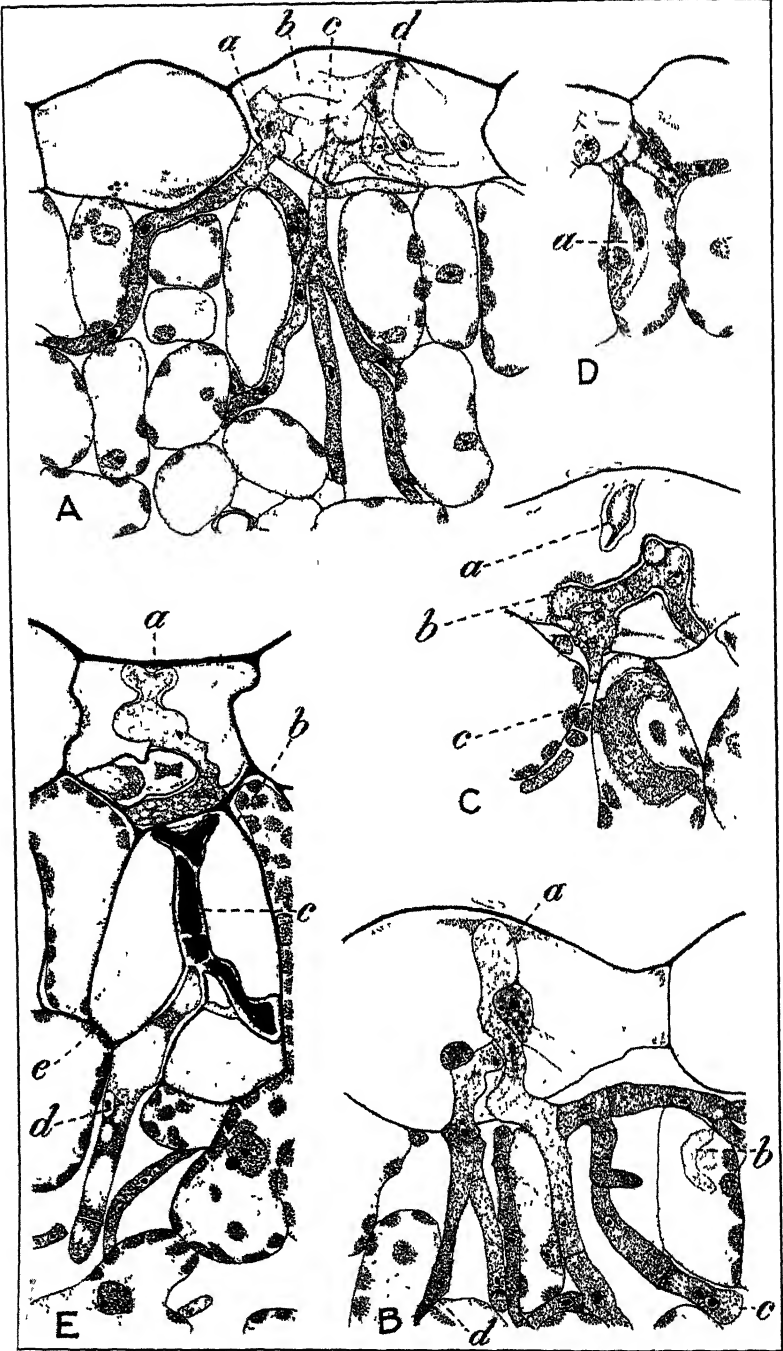
By the seventh day another change usually is noticeable. Plate 2, C,  $a$  and  $b$ , shows the primary hypha (divided in sectioning) coated by a layer of material staining like the host cell walls and possibly serving as a defense of the host against the intruder.

This sheath persists for a time after the primary hypha that it incloses has disintegrated, and becomes of value in later studies. Whether an infected area is of monosporidial or of multisporidial origin may be determined by the presence of one or of more than one sheath in the cells of the upper epidermis.

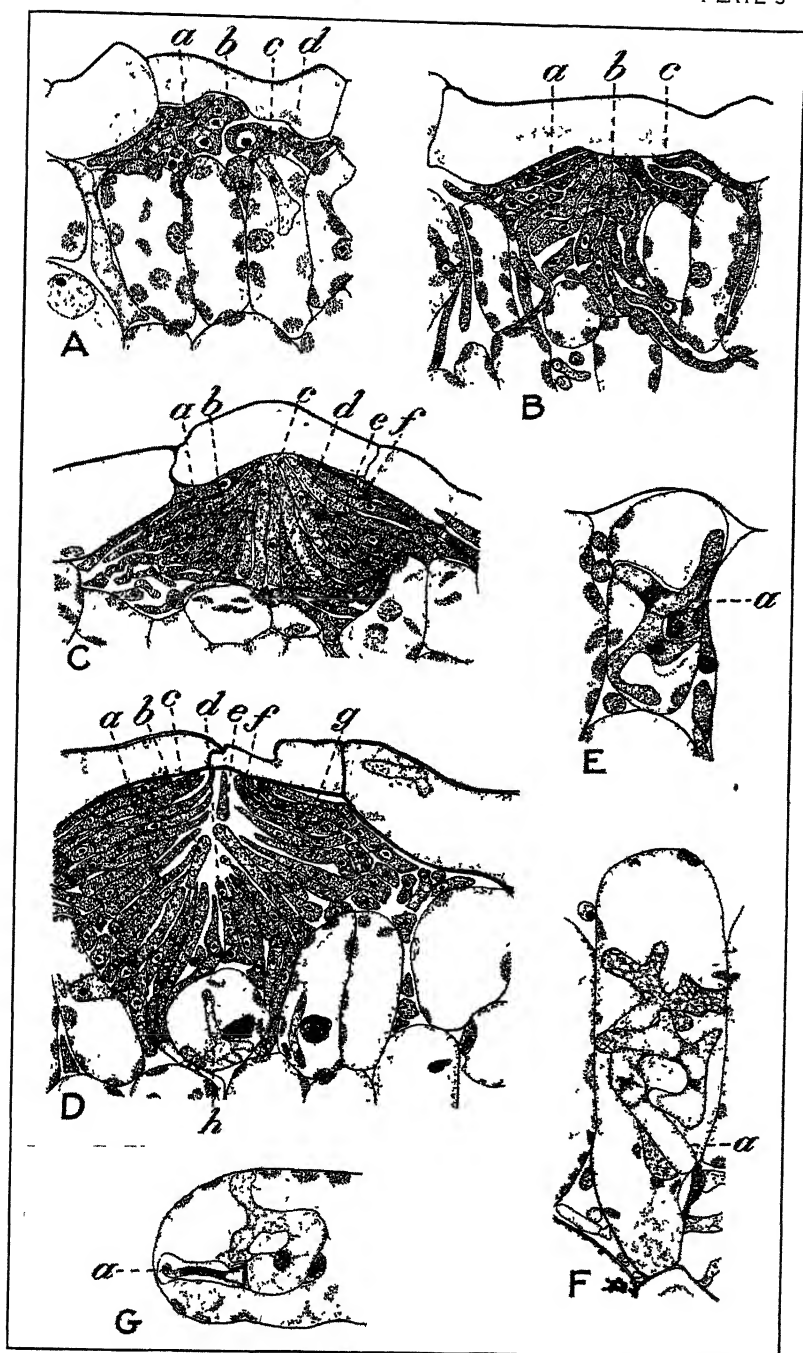
After leaving the epidermal cell the hyphae make their way down to the large air spaces of the spongy mesophyll. Ordinarily, they follow intercellular channels, passing through the natural spaces between palisade cells or, in case of need, forcing a passage by splitting

#### EXPLANATORY LEGEND FOR PLATE 2

- A—Four-day infection with primary hypha ( $b$  and  $d$ ) and branches forming intercellular mycelium ( $a$  and  $c$ )  $\times 1,020$   
 B—Seven-day infection with primary hypha ( $a$ ), mycelium ( $c$  and  $d$ ), and haustorium ( $b$ )  $\times 1,020$ .  
 C—Seven-day infection with degenerating hypha ( $a$  and  $b$ ) unsheathed in cell-wall materials, and haustorium ( $c$ )  $\times 1,020$   
 D—Three-cell haustorium or hypha ( $a$ ) in palisade cell of 4-day infection  $\times 1,020$   
 E—Eleven-day infection with remnant of primary hypha ( $a$ ) giving rise to the hypha  $c-d$  which passes through the palisade cell  $b-c$ .  $\times 1,020$



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in two the wall between two palisade cells (Pl 1, D<sub>1</sub>, *d*, and E, *a* and *d*)

More rarely a hypha will attempt to grow through a palisade cell. Plate 2, D, *a*, shows a 3-cell hypha in a palisade cell. It is intermediate in character between a hypha and a haustorium and perhaps would not have grown further. In Plate 2, E (from an 11-day infection), however, the attempt to grow through a palisade cell has been successful. From the primary hypha (*a*), now disintegrated and misshapen, a hypha (*c*) entered the palisade cell (*b-e*), grew down through it and then out at *d*. The palisade cell and much of the hypha within it are dead.

The developing mycelium extracts food from the host cells by means of haustoria. These may be small, 1-cell, and unbranched, or they may be larger and more complicated. Examples of simpler haustoria are shown in Plate 2, C, *c*, and B, *b*, and in Plate 3, A, *c*, and D, *h*. Larger, branched haustoria consisting of more than one cell are shown in Plate 3, E, *a*, F, *a*, and G, *a*. In old age the haustoria, like the primary hyphae, often become insheathed in materials staining like host cell walls.

#### SPERMOGONIA AND RECEPTIVE HYPHAE

After six or seven days of vegetative growth, during which the mycelium spreads through the air spaces of the mesophyll, reproductive activity sets in. Hyphae grow to both the upper and the lower epidermis, giving rise there to spermogonia (pycnia) and to receptive hyphae.

When a spermogonium is to form, hyphae grow to the epidermis (upper or lower) and form a small compact group of dense, well-nourished cells in contact with the epidermal layer. Plate 3, A, shows an early stage of this process. Even at this early stage there is a beginning of organization, for hyphae at *a* and *d* are slanting up toward a common center at *b*.

Soon after this a more definite arrangement of hyphae is evident. In a median section through a young spermogonium from a 7-day infection (pl 3, B), the hyphae growing in from all sides are definitely centered at *b*. The slender hyphae at *a* and *c*, close to the epidermis and nearly parallel to it, will become the paraphyses. The thick upright hyphae at *b* will become the buffer cells that lift the epidermis and resist its pressure.

Plate 3, C, shows a later stage of development. The buffer cells at *c* have become heavy upright columnar cells pressed against the epidermis. Their cytoplasm has taken on the open alveolar structure characteristic of buffer cells. Growing in between the buffer cells from the base and sides at *b*, *d*, and *e* are the first of the young spermatophores that later will produce spermatia. At *a* and *f* are the young

#### EXPLANATORY LEGEND FOR PLATE 3

- A —Detail of 7-day infection showing beginning of formation of spermogonium at *a*, *b*, and *d*. Haustorium at *c*. × 640  
 B —Slightly older spermogonium from 7-day infection. Young buffer cells at *b*, and paraphyses at *a* and *c*. × 640  
 C —Growing spermogonium with buffer cells at *c*, paraphyses at *a* and *f*, and spermatophores at *b*, *d*, and *e*. × 640  
 D —Older spermogonium from 8-day infection with buffer cells at *b* and *e*, spermatophores at *a*, *d*, and *g*, and paraphyses at *c* and *f*. Central cavity forming. Haustorium at *h*. × 640  
 E —Two-cell haustorium at *a* from 7-day infection. × 1,020  
 F —Much-branched haustorium (*a*) from 8-day infection. × 1,020  
 G —Decadent haustorium (*a*) from 9-day infection. × 1,020

paraphyses All these hyphae, whether basal or lateral in origin, are focused on the central point (*c*)

A rapid increase in the number of spermatophores follows These are slender tapering cells that grow in from the base and side walls of the spermogonium The interpolation of these new cells between the buffer cells (*pl 3, D*) causes a rapid expansion of the spermogonium and arches it out into a spherical mass that crushes the adjoining host cells as it grows As the spermogonium rounds out, a central cavity forms within it, lined by the tips of the spermatophores (*a, d, and g*) The buffer cells (*b and e*), released from pressure against the epidermis, project into the central cavity. They produce no spores and soon wither and die

Plate 4, A, shows a newly opened spermogonium from a 9-day infection The paraphyses (*c and d*) have pierced the epidermis forming the ostiole and are growing out through the opening. The basal part of the spermogonium forms a hemispherical shell of radially arranged spermatophores, among which is still to be found an occasional buffer cell (*b and f*). Spores formed by the spermatophores fill the central cavity (*e*) and are moving out through the ostiole The spores are exuded in a viscous liquid, the nectar, which has a distinctly flowerlike odor The exudate is not abundant, but serves by its perfume to attract insects

The first spermogonia usually mature and open seven or eight days after inoculation The number of spermogonia is highly variable, however In 9-day infections there may be as many as a dozen open spermogonia, or only two or three, or none whatever

In *Puccinia triticina* the spermogonia are about equally distributed between the upper and lower surfaces of the leaf A count shows that of 100 spermogonia in 11-day infections, 46 opened on the upper surface of the leaf and 54 on the lower

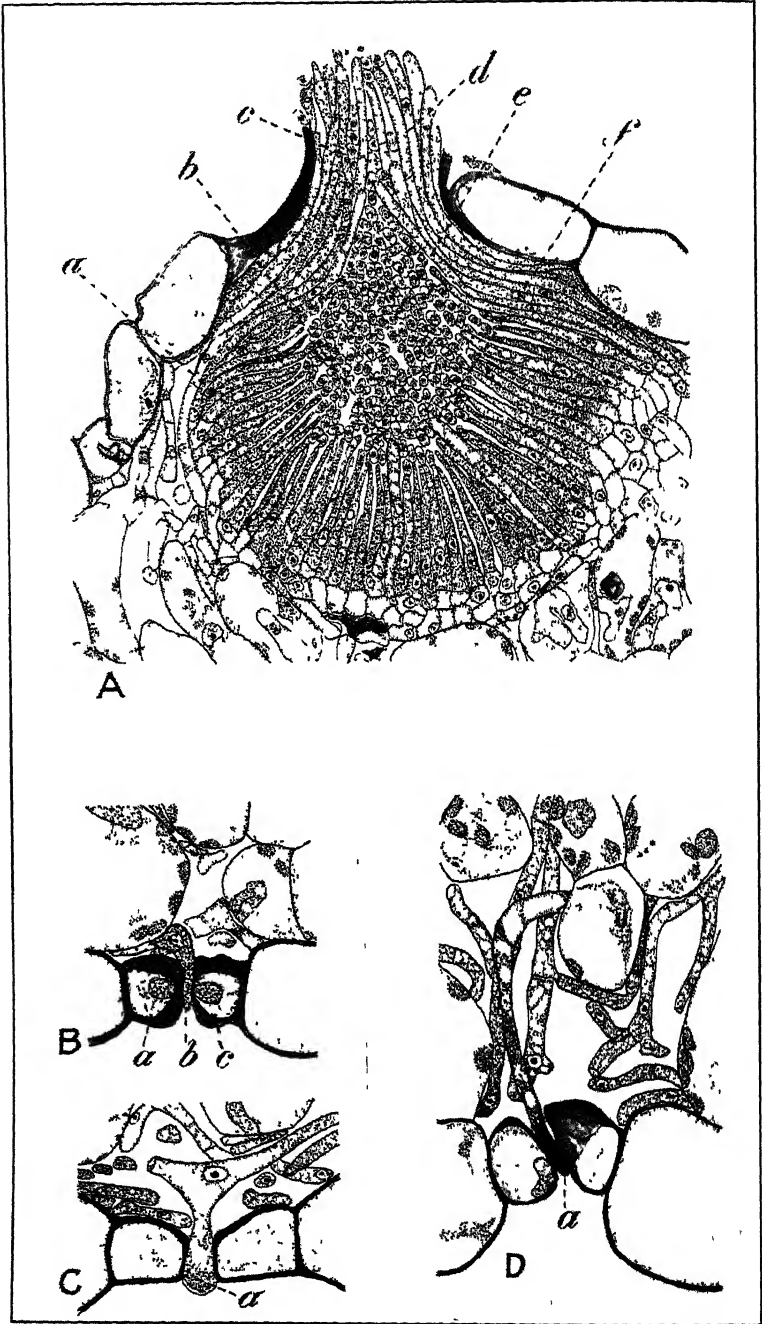
While spermogonia are developing, certain hyphae are growing into stomatal apertures or forcing a passageway between epidermal cells of the leaf. Since these hyphae reach the surface of the leaf and serve to receive the spermatial nuclei, the terms "emergent hyphae" or "receptive hyphae" have been applied to them

In the stomata, which in *Thalictrum* are on the lower surface of the leaf, hyphae of the fungus are to be found inserted into stomatal apertures. Plate 4, B, shows an early step in this process A vigorous young wedge-shaped hypha (*b*) is pushing between the two guard cells (*a and c*) of the stoma. Whether such a hypha can force entrance into a closed stoma or must await its natural opening is not known; the latter seems probable.

Plate 4, D, shows another hypha The stoma here is cut obliquely In this instance several other hyphae are growing toward the same stoma. These hyphae, like the mycelium producing them, are composed of uninucleate cells Plate 4, C, shows the further development of a cell in a stoma The inserted hypha has grown larger and its broadened tip has reached the lower surface of the leaf.

#### EXPLANATORY LEGEND FOR PLATE 4

- A — Mature spermogonium from 9-day infection Paraphyses (*c and d*) protruding through ostiole Radially arranged spermatophores (*a*) have formed spores filling central cavity (*e*) Buffer cells (*b and f*)  
 × 640  
 B — Young receptive hypha (*b*) thrust into stoma (*a-c*), 9-day infection × 1,020  
 C — Older receptive hypha (*a*) in stoma of 11-day infection × 1,020  
 D — Receptive hypha (*a*) in stoma of 9-day infection × 1,020



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11 single infections, all were sterile on the twenty-third day, although at that time 9 out of the 10 multiples on the same plant bore open aecia. On another host plant 19 out of 21 singles were sterile on the twenty-sixth day, at which time 4 out of the 5 doubles and the 4 multiples were producing aeciospores. On still another plant 19 out of 21 singles were sterile on the forty-second day after inoculation, at which time the 8 doubles and the 10 multiples were fertile. On yet another plant the 15 singles were still sterile on the forty-eighth day. The extreme case was a plant bearing 10 sterile singles 64 days old. At this time the infections and the leaves bearing them were dying.

The microscope shows that the majority of the isolated single infections bear both spermatogonia and receptive hyphae in varying proportions, but the greenhouse studies show that the spermatia of a given rust plant can not fertilize the same plant, it is self-sterile.

More than the expected percentage of doubles produce aeciospores. In a heterothallic rust which is strictly bisexual, half of the infections would be (+) and half (-). In a random assortment half of the doubles would consist of a (+) and a (-), while the other half would consist of either two (+) or two (-). In other words, in 50 per cent of the doubles, (+) and (-) meet, and the combination should be fertile, while in the other 50 per cent, both members are of the same sex, and the combination should remain sterile. An actual count of doubles of ages ranging from 24 to 29 days gives 11 sterile and 31 fertile, or about 26 per cent sterile and 74 per cent fertile. A small amount of this is to be ascribed to accidental outside fertilization. In the data on single infections cited above, 4 out of 74, or about 5 per cent, bore aeciospores. Allowing a similar margin here for accidental fertilization, there is still a large surplus over the expected number of fertile doubles.

#### THE ISOLATED INFECTION

Material was available for a detailed study of the later history of the infection in which fertilization does not take place.

Reference has already been made (pl. 4, B, C, and D) to the emergent hyphae thrust between guard cells of stomata, and to other hyphae (pl. 6, A) that force a passageway between cells of the upper epidermis.

A hypha in a stomatal aperture is short-lived. Perhaps the exposure to the drier air outside of the leaf kills it. Perhaps, when the stoma attempts to close in the course of its daily stomatal movements, the hypha gets crushed. At any rate, the appearance of the inserted hypha soon changes. In Plate 5, A, the hypha (b), which grew down between the guard cells (a and c), takes the deep red stain characteristic of dead or dying protoplasm.

But this does not end the matter. Other hyphae have been massing in the air space above the stoma, and branches from these push down into the stoma alongside the first. Each stomatal hypha, in turn, dies and shrinks and another takes its place. In Plate 5, B, which represents a longitudinal section through a stoma of an 11-day sterile infection, five hyphae in succession have projected into and through the stomatal aperture. The two oldest (b and e) are dead and two others (a and d) are dying. Only the youngest (c), at the center of the group, is still fresh and vigorous. Plate 5, C, shows a

A less conspicuous formation of "emergent hyphae" occurs at the upper epidermis of the leaf. Plate 6, A, represents a detail from an 11-day infection showing the upper epidermis (*a*) and the palisade layer (*c*) beneath it. Between the two cell layers at *d* is a scant subepidermal mycelial growth, and from this at *b* a hypha has grown up between the epidermal cells to the upper surface of the leaf. It has not pierced the cuticle at the outer epidermal wall.

A few of these hyphae emerge at the upper and lower surfaces when infections are 7 days old and many more by the eighth or ninth day, but the development is not uniform. A survey of 9-day infections shows that an infection which bears numerous spermogonia is apt to have relatively few receptive hyphae, while an infection with no spermogonia shows a rich development of these hyphae.

#### GREENHOUSE NOTES

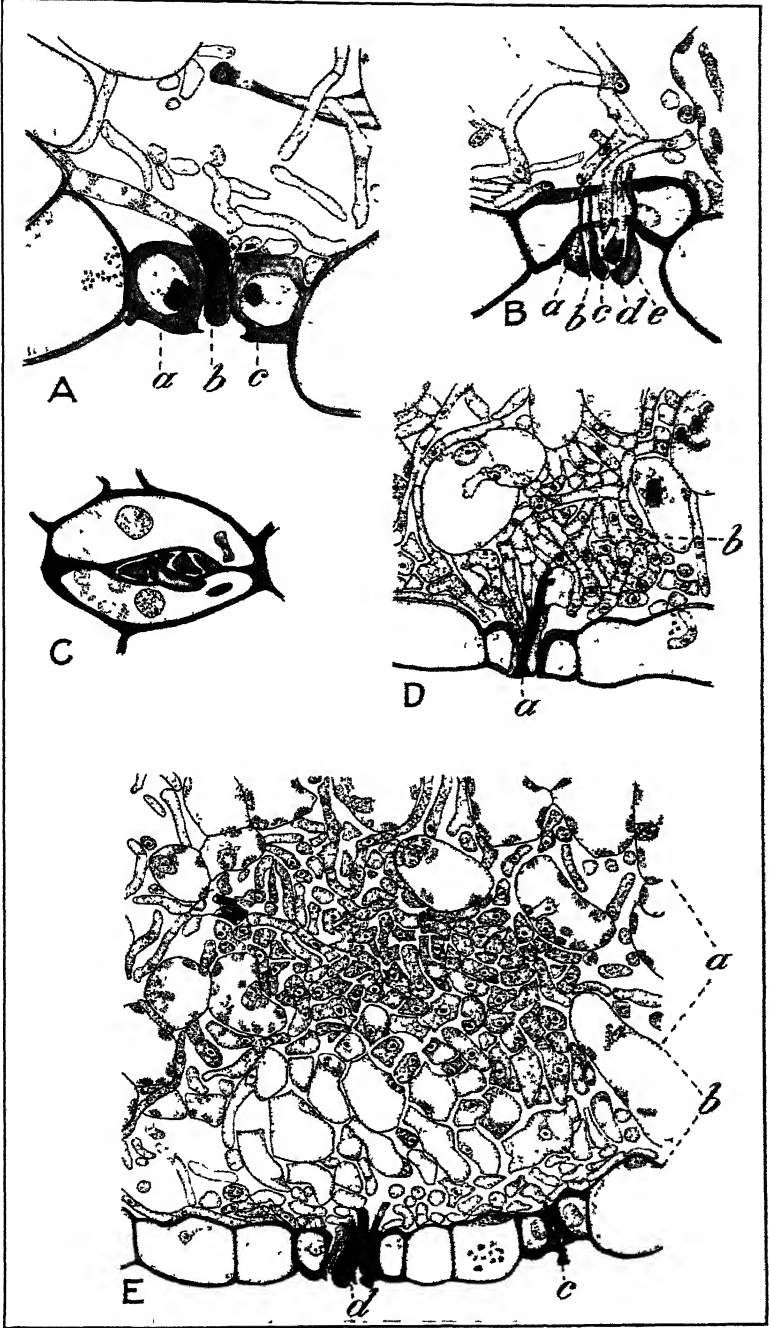
Living infections have been studied in the greenhouse. The first macroscopic indication of the rust is the "fleck," a minute whitish spot on the leaf, less than a millimeter in diameter. Flecking begins six or seven days after inoculation. A day or two later both the upper and the lower surfaces of the infected area are yellow with the exudate, or nectar, from spermogonia, which is distinctly fragrant.

The infections grow rapidly. The size attained by an infection varies with the age and vigor of the leaf and with the number of infections on the leaf. Where infections are crowded together the individual infection remains small. The greatest size of the individual infection and the greatest deformation of host tissues occur on young tender leaves that carry one or only a few infections. Infected petioles of young leaves become hypertrophied, attaining as much as three times the normal diameter. On a young leaf blade the maximum diameter of an isolated infection is 8 or 10 mm. The infections often become irregular in outline, the spread being limited more or less by the larger veins. The leaf becomes distorted and thickened in the infected area and bulges upward. These hypertrophied tissues are under tension and, when slit for fixing, the strips spring apart.

Owing to the fact that relatively few host plants have been available for this work, it was necessary to fix material from time to time from the plants on which the greenhouse counts were made, therefore only a part of these infections have been followed through to old age. In the greenhouse records, infections are classified as singles (isolated infections), doubles (two in contact), and multiples (several confluent infections).

The greenhouse studies of the rust indicate that *Puccinia triticina* is heterothallic, for the isolated infection usually remains haploid and produces no aeciospores. An occasional single infection develops open aecia, perhaps fertilized by nectar carried by a thrip or a red spider or by chance contact with the nectar on another infected leaf beneath. Some of the infections situated fairly close together are singles at first, but during later growth become doubles or even multiples through confluence. These may develop belated aecia.

Nearly all the multiples and a large majority of the doubles produce open aecia between the eighteenth and the twenty-second day after inoculation. On a few of the inoculated plants the infections were studied and counted for a considerable period. On one plant with



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tangential (surface) view of such a stoma into which, as before, five hyphae in turn have grown. It is not uncommon to find one to six hyphae in almost every stoma in the infected area.

As stated earlier, the number of these stomatal hyphae in an infection varies more or less inversely as the number of spermogonia present. There are few in infections with numerous spermogonia and a great many in infections with no spermogonia.

There are apparent exceptions to this. In one infected area studied, which at first looked like one continuous infection, both spermogonia and emergent hyphae were abundant. But careful study of the upper epidermis showed the presence of four primary hyphae at different points (one of these is shown in pl 2, E), so the apparently simple infection consisted really of four closely interwoven infections. It would be premature to say, however, that all such cases are of multi-sporidial origin. Only a long, careful study could determine that. As would be expected in the case cited above, fertilization had occurred at one or two points.

The amount of fungous growth back of a stoma at the time hyphae are pushing into the stomatal opening varies considerably. In Plate 5, B, the substomatal air space behind the stoma is but loosely threaded by scattered hyphae. In Plate 5, D (drawn at lower magnification than the preceding), where fresh hyphae are still being thrust into the stoma, the substomatal cavity (pl 5, D, b) is filled with a dense fungous growth. It is noteworthy that the entire growth at this stage centers on the occupied stoma. Fertilization has not taken place, the cells are all uninucleate. It is at these points that aecia, fertile or sterile, arise.

In the absence of fertilization, this haploid substomatal growth continues for some time. Plate 5, E, shows a later stage in the development of the sterile aecium of an 11-day infection. The fungous mass has attained considerable size and has undergone the first differentiation into an upper half (a), of short, thick cells with dense cytoplasm and large nuclei, and a lower half (b), of large, loosely spaced cells with open vacuolate contents. In both parts the hyphae have become more or less disarticulated, each cell rounding up by itself. There are two stomata (c and d), both occupied by hyphae already dead. Due to the expansion of the cells and the disarticulation of the hyphae these emergent hyphae can no longer be traced into the body of the aecium. Fertilization did not take place; the aecium is still haploid.

Reference has already been made to the occasional formation of a thin layer of mycelial growth beneath the upper epidermis of the leaf and to the hyphae that push up from it between the epidermal cells. These are of much less frequent occurrence than are the hyphae within the stomata.

Under certain circumstances the development at the upper epidermis is much greater. In studying the living infections in the

#### EXPLANATORY LEGEND FOR PLATE 5

- A —Dying stomatal hypha (b) between the guard cells (a and c) in a 9-day infection.  $\times 1,020$   
 B —Longitudinal section of a stoma of an 11-day infection with 5 receptive hyphae, 2 dead (b and e), 2 dying (a and d), and 1 living (c).  $\times 1,020$   
 C —Surface (tangential) view of stoma from an 11-day infection. Within the stomatal aperture are 5 receptive hyphae, all dead.  $\times 1,020$   
 D —Nine-day infection. Stoma (a) filled with receptive hyphae, back of which is the young aecial primordium (b).  $\times 640$   
 E —Older sterile aecium from an 11-day infection, showing differentiation into a denser upper portion (a) and a more open lower half (b). Occupied stomata (c and d).  $\times 640$

greenhouse it was noted that infections on young, tender leaves make a more luxuriant growth and deform the leaves more than do infections on older, tougher leaves. Sections through the infections on young leaves show that a fairly massive fungous growth may form between the epidermis and the palisade layer. Plate 6, C, shows semidiagrammatically a section through a 9-day infection. There are no spermatogonia in this infection. Under the upper epidermis is a continuous growth of hyphae, which at certain points (*b*, *c*, and *d*) has formed an upright palisade of parallel hyphae pushing between the epidermal cells. At *a* is a side section through a similar mass.

Plate 6, D, represents one of these (C, *b*) enlarged, showing the group of upright hyphae from *b* to *c* and later subsidiary attempts to pierce the epidermis at *a* and *e*. On the surface of the leaf is a dark-staining mass (*d*), presumably exuded either by the fungus or by crushed host cells.

In only one or two cases were similar but smaller subepidermal growths found at the lower surface of the leaf. One of these (pl. 6, E) occurred opposite a leaf vein where the absence of stomata prevented the formation of the ordinary receptive hyphae in stomata. Here, as before, several hyphae have squeezed in between epidermal cells, and others are growing in the same direction. The whole structure (pl. 6, E) is focused at *a*, where the hyphae have separated two epidermal cells. And here, as in the other cases, the cuticle of the outer epidermal wall is still intact, so that these hyphae are not really exposed to the outside air.

The nature of these anomalous structures is unknown, but since all gradations can be found, from the simple hypha between epidermal cells in Plate 6, A, to the more massive growth in Plate 6, D, it is probable that they are of the same nature. They occur in infections without spermatogonia but with abundant hyphae in the stomata of the lower epidermis. The presumption is that the hyphae between epidermal cells, whether occurring singly or in groups, are receptive in nature. The similarity in structure between the group of hyphae in Plate 6, E, and the aecial primordium shown in Plate 5, D, is unmistakable.

The similarity stops at this point. No case has been noted of a sterile aecium developing either in connection with a single hypha between epidermal cells (pl. 6, A) or adjoining the more massive structures (D and E). In the absence of fertilization these structures, once formed, persist without developing further, so far as known, and become gradually decadent. Plate 6, B, shows a small group of these hyphae between epidermal cells from a 22-day infection. The cells are nearly dead and still haploid, and the mycelium beneath consists of a loose tangle of ordinary hyphae.

The early stages of growth of the sterile aecium (pl. 5, D) are centered on the receptive hyphae in a stoma. In the later development

#### EXPLANATORY LEGEND FOR PLATE 6

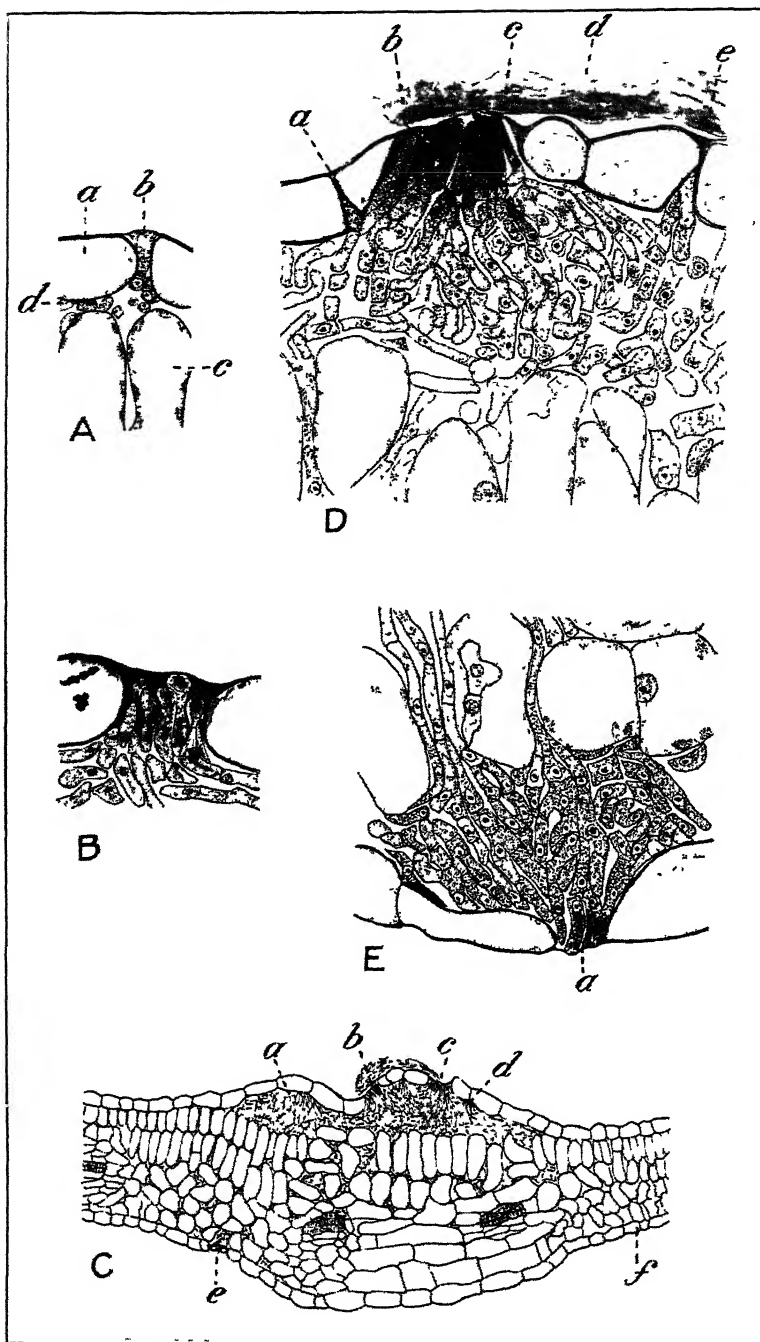
A—Eleven-day infection. Between the upper epidermis (*a*) and the palisade layer (*c*) is a scant mycelial growth (*d*) from which a hypha (*b*) has grown up between epidermal cells.  $\times 1,020$

B—A group of similar cells, now dying, between epidermal cells in a 22-day infection.  $\times 640$

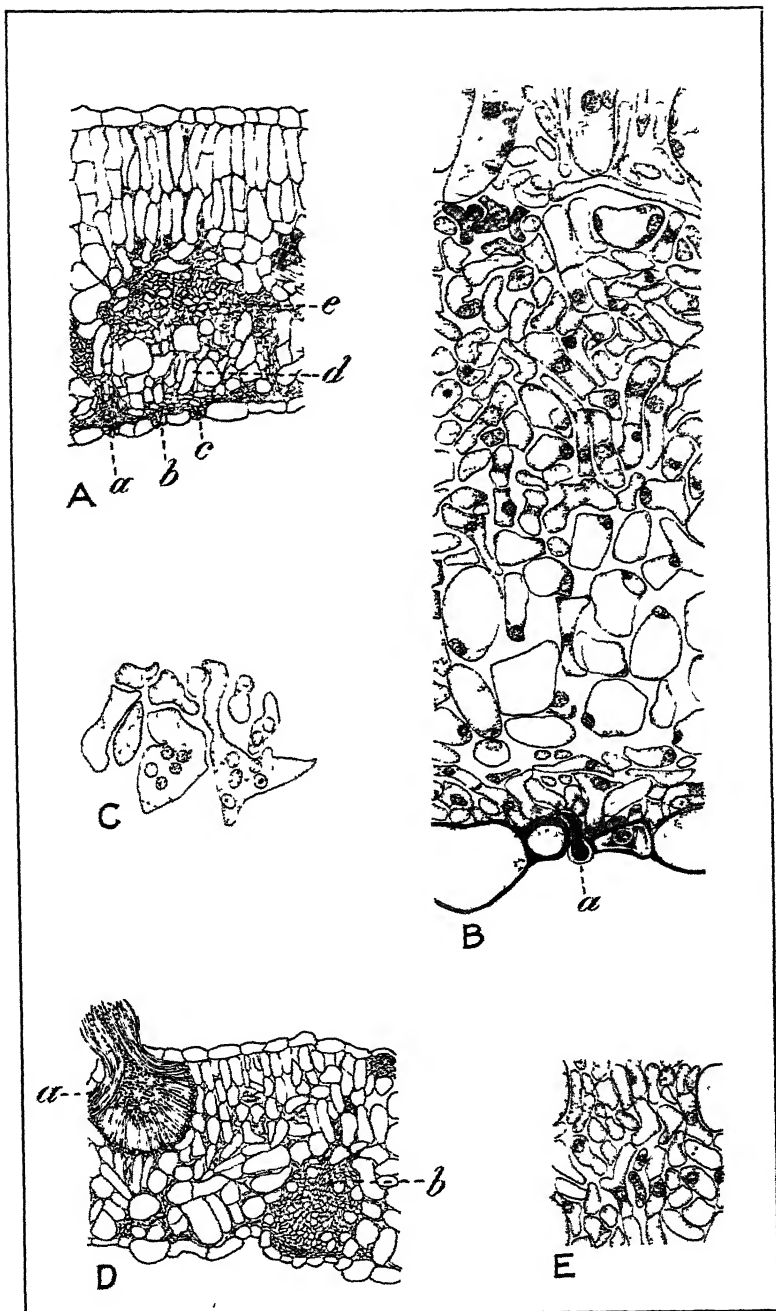
C—Diagram of leaf with 9-day infection showing subepidermal growth, bearing groups of upright hyphae between epidermal cells (*a*, *b*, *c*, and *d*). Receptive hyphae in stoma (*e*). Normal thickness of leaf (*f*).  $\times 115$

D—Group of upright hyphae (*b-c*) growing between epidermal cells of 9-day infection and subsidiary hyphae that attempted to grow between epidermal cells (*a* and *e*). Exudate (*d*).  $\times 640$

E—Similar group of hyphae emerging between cells of lower epidermis (*a*) next to a leaf vein in a 9-day infection.  $\times 640$



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the fungous growth above adjoining stomata may become confluent, forming one aecium. This is usually the case in the larger aecia. Plate 5, E, shows two occupied stomata (*c* and *d*). Plate 7, A, shows an aecium, from a 15-day sterile infection, with three sets of receptive hyphae in the stomata (*a*, *b*, and *c*) and others in adjoining sections.

The leaf tissues in this case have become hypertrophied. Plates 6, C, and 7, A, are drawn at the same magnification. The leaf now (pl. 7, A) is between two and three times the normal thickness as seen in Plate 6, C, *f*. The aecium (*d-e*) extends through more than half the thickness of the overgrown leaf.

The aecium is still haploid. All its cells are uninucleate (pl. 7, A), both the smaller ones in the upper region (*e*) and the large ones in the more open area below (*d*).

Plate 7, B, shows, at higher magnification, a strip through a large sterile aecium of a 30-day infection. It is still living, but decadent. Remnants of the stomatal hyphae may still be seen at *a*. The cells are uninucleate throughout.

In the great majority of the sterile aecia examined the cells remain uninucleate until death, but a few exceptions occur. In Plate 7, C, which shows a detail from a sterile aecium with every evidence of deterioration, there are a few multinucleate cells. Even these have an impoverished appearance, with scant cytoplasm and large vacuoles.

The amount of hypertrophy of the leaf and the size attained by the sterile aecium vary greatly in different infections. Plate 7, D, from a 22-day infection, shows a cross section of a leaf drawn at the same magnification as that shown in Plate 7, A. The contrast between the two in respect to the thickness of the leaves and the size of the aecia is marked. In one (pl. 7, A), an infection without spermogonia, the leaf is much thickened and the aecia are large; in the other (pl. 7, D), an infection bearing spermogonia, the leaf is less thickened and the sterile aecia are relatively small. The aecia here (D, *b*) are not only smaller but also less differentiated. A bit of the aecium shown in Plate 7, D, is enlarged in Plate 7, E.

In the end the aecia of the isolated infection die without producing spores. Plate 8, A, shows a small dead aecium of a 42-day infection. Even here the remnants of receptive hyphae can be seen at *a*.

It was noted earlier that infections of *Puccinia tritici* vary widely in the proportion of spermogonia to receptive hyphae. This difference persists into old age. One 42-day infection, a section of which is shown in diagram (pl. 8, B), bore 103 spermogonia, and only 23 small sterile aecia. Another 42-day infection of the same lot of material, shown in diagram (pl. 8, C), bore over 200 sterile aecia but had no spermogonia whatever. A comparison of the two diagrams shows other differences. They are drawn at the same magnification ( $\times 33$ ). In the spermogonial infection the hypertrophy of the leaf is slight; in the aecial infection it is much greater. The aecia of the spermogonial infection (pl. 8, B, *f* and *h*) are minute; those of the aecial infection

#### EXPLANATORY LEGEND FOR PLATE 7

- A.—Semidiagrammatic drawing of cross section of a hypertrophied leaf bearing large sterile aecium, *d-e*. Stomata with emergent hyphae (*a*, *b*, and *c*), 15-day infection.  $\times 115$   
 B.—Narrow strip through a large sterile aecium from 30-day infection. Receptive hyphae (*a*).  $\times 640$   
 C.—Multinucleate cells from sterile aecium from 22-day infection.  $\times 640$   
 D.—Diagram showing cross section of leaf with spermogonium (*a*) and sterile aecium (*b*).  $\times 115$   
 E.—Enlarged detail from aecium in D.  $\times 640$ .

(pl 8, C, *a*, *b*, *c*, *d*, *e*, and *f*) are variable in size, but on an average are several times as large as in the spermogonial infection.

The spermogonia of the old sterile infection (pl 8, B, *a*, *b*, *c*, *d*, *e*, and *g*) are for the most part nonfunctional, but a few are still active

#### THE FERTILE INFECTION

The surface of the *Thalictrum* leaf is waxy. When infected leaves of *Thalictrum* are fixed, the external spermatia wash off, either in the fixing fluid or during the subsequent washing and dehydrating. In slides made from paraffin sections, spermatia of *Puccinia triticina* are rarely seen except inside the spermogonium or adhering to its paraphyses. On this account the actual entering of spermatial nuclei into receptive hyphae has not been seen. Perhaps it could be done by making free-hand sections of living infections.

After a spermatial nucleus has entered the hypha, however, it is secure against the processes of the technic and can be found in the stained sections. That nuclei do appear in the receptive hyphae, often in considerable numbers, after the application of spermatia to the surface of an infection is amply demonstrated.

In the unfertilized infection (pls 5, 6, 7, and 8) the emergent hyphae and the sterile aecia of different ages are, in general, composed throughout of uninucleate cells. Upon being fertilized the multinucleate condition is found, first, in the hyphae at the epidermis, and later at points more remote from the surfaces of the leaf.

Plate 9 shows details from newly fertilized infections. In Plate 9, B, is a longitudinal section of a stoma in which there is one stomatal hypha (*b*) containing two nuclei, and another (*a*) containing four nuclei. In Plate 9, C, fertilization occurred at two stomata that were contributory to the same aecium. The receptive hypha *a* contains 3 nuclei, *b* has 6, and *c* has 5. In cases like that shown in Plate 9, A, it is uncertain whether the sporophytic cells *a* and *b*, some distance above the stoma, are derived from a hypha at the stoma *c* or from some other stoma near by.

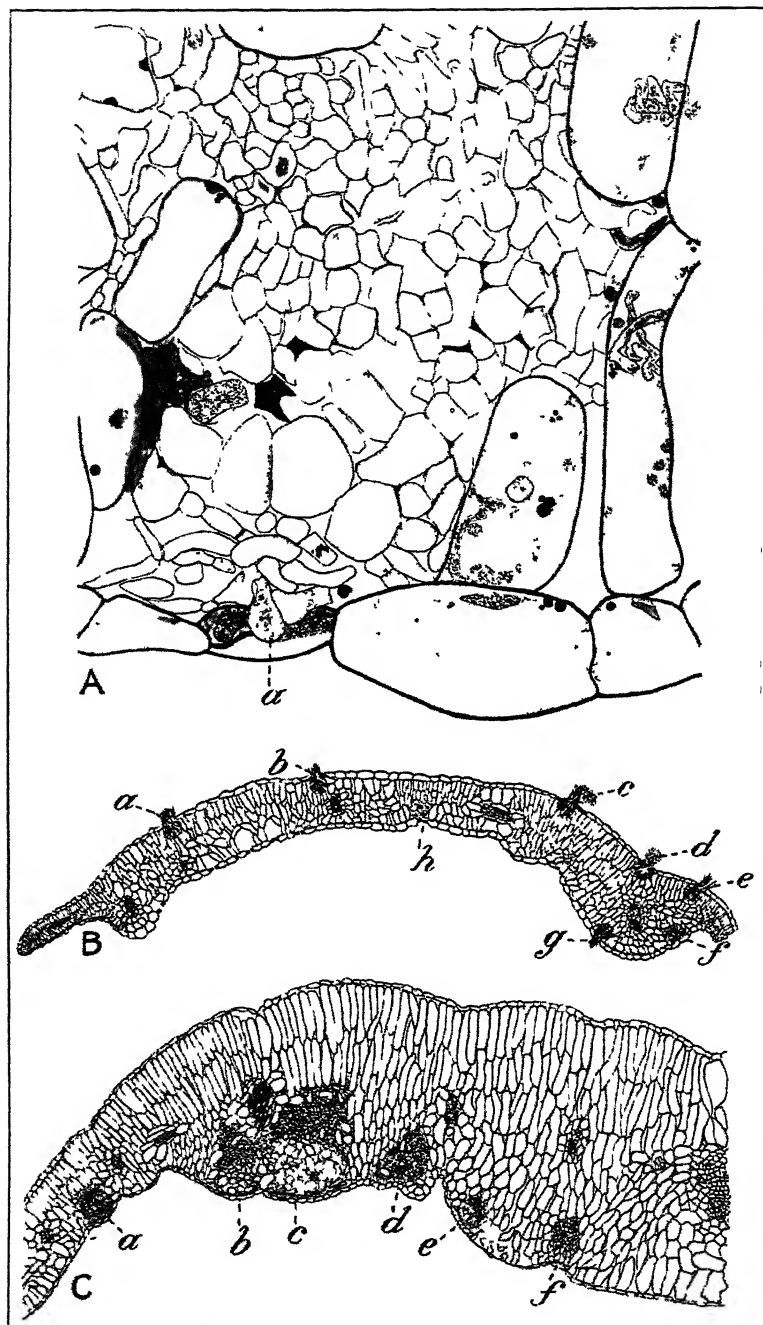
Plate 9, D, *a*, shows a hypha the tip of which reached a stoma in an adjoining section at a position corresponding to the point *b*. It contains 11 nuclei. The number of nuclei entering an emergent hypha would appear to be limited by the available supply of spermatia outside rather than by any saturation point within the hypha. Of course it is possible, but less likely, that only one nucleus enters, which immediately divides rapidly.

Sometimes accessory points of fertilization are formed by hyphae between cells of the lower epidermis. In Plate 9, E, is a group of emergent hyphae (*a*, *b*, and *c*) not unlike the groups at the stomata in appearance. One contains 2, one 4, and one 6 nuclei. So far as the microscope reveals, both the cuticle of the leaf and the walls of the hyphae themselves are still intact. The entrance of the spermatial nucleus has not left a visible pore.

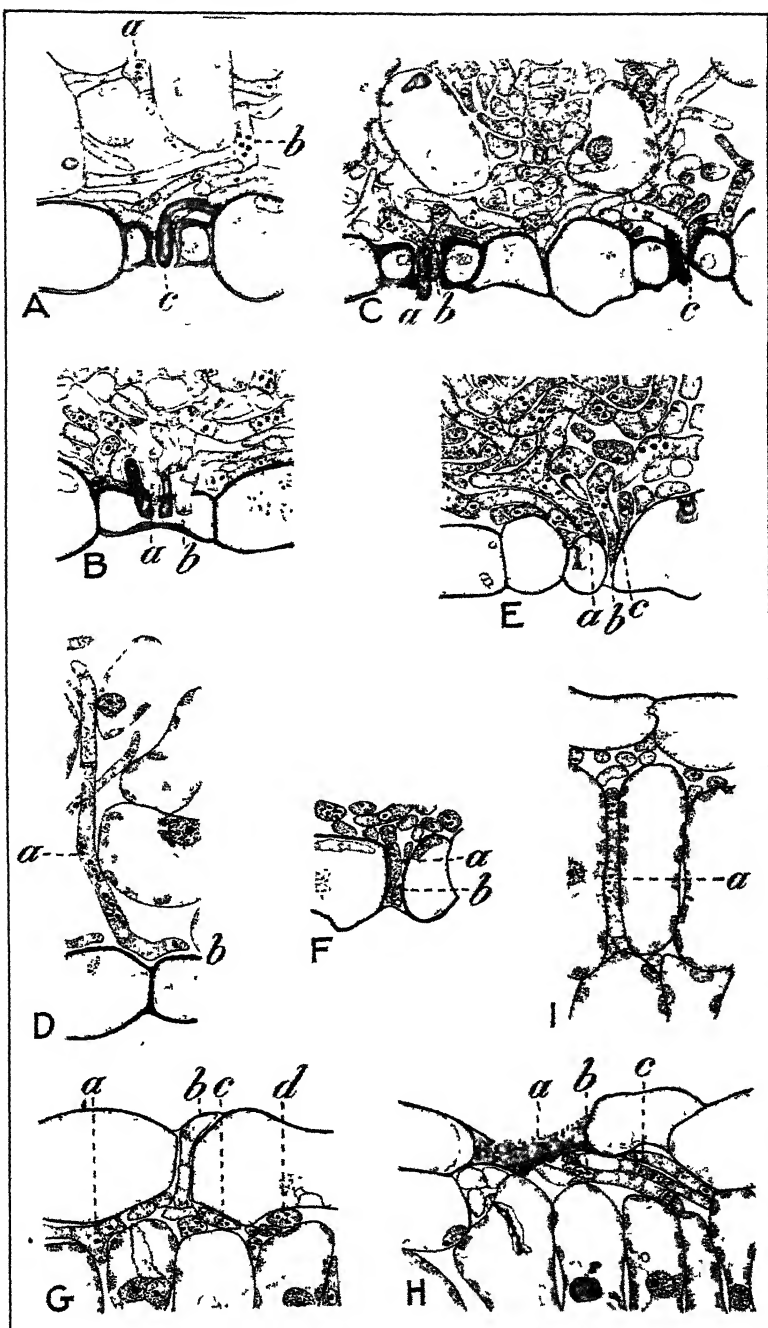
Plate 9, F, *a* and *b*, shows a similar instance. In this case the hypha *b* is flattened against the cuticle and two very minute nuclei are just inside the cell, perhaps having just entered.

#### EXPLANATORY LEGEND FOR PLATE 8

- A—Small, dead, sterile aecium from 42-day infection. Remnant of receptive hyphae (*a*)  $\times 640$   
 B.—Diagram of 42-day spermogonial infection with spermogonia (*a*, *b*, *c*, *d*, *e*, and *g*) and small sterile aecia (*f* and *h*)  $\times 33$   
 C.—Diagram of 42-day aecial infection, showing large sterile aecia (*a*, *b*, *c*, *d*, *e*, and *f*)  $\times 33$



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Less frequently, fertilization occurs at the upper epidermis. Plate 9, G, shows a hypha (*b*) that grew up between epidermal cells and pushed along for a short distance beneath the cuticle. Fertilization occurred, followed by sporophytic growth from the base of the cell. There are still 2 nuclei in the base of the hypha, and from 2 to 5 nuclei in near-by cells (*a*, *c*, and *d*).

Plate 9, H, shows an unusual case. Some minor injury to the leaf killed a single epidermal cell (*a*), and there are a few sporophytic cells (*b* and *c*) just beneath the dead cell. Apparently, fertilization was effected through the dead cell. It may be that the outside air, entering through the dead, withered host cell, served as the stimulus that attracted hyphae to this point. Perhaps it is a positive aerotropic response that brings all the emergent hyphae to the leaf surface, whether in stomata, between epidermal cells, or at dead epidermal cells.

No aecial primordia have been found beneath the upper epidermis in these cases. The sporophytic hyphae grow down between the palisade cells and out into the spongy mesophyll. In Plate 9, G, the hypha at *a* is just pushing a branch into the space between two palisade cells. In Plate 9, I, the hypha at *a*, containing five nuclei, has almost reached the mesophyll. Other sporophytic hyphae have been found traversing the air spaces of the spongy tissue and above young aecia. No one sporophytic hypha can be traced from a receptive hypha at the upper surface to an aecium, but the probability is that the fertilizations at the upper leaf surface contribute to the sporophytic growth in the aecia beneath them, either by growth of new sporophytic hyphae or by progressive "diploidization" of the already existing mycelium, accomplished by successive nuclear divisions and migrations.

Soon after the introduction of spermatial nuclei into the receptive hyphae the haploid component of the aecial primordium becomes permeated throughout by scattered cells containing two or more nuclei (Pl 10, A). The question arises as to the mode of distribution of the sporophytic generation from its point or points of origin.

Plate 10, B, shows a young aecium in which the haploid hyphae are still so loosely spaced that unusual opportunity is afforded for tracing out the course of individual hyphae. A group of hyphae centers on the stoma at *d*. One of these hyphae, which grew down from *a*, is now dead throughout most of its length, but near the stoma has given rise to a sturdy diploid hypha that has grown up to *e*, at which point it passes out of the plane of the section. Another diploid hypha growing upward from the receptive hyphae at the stoma is unbranched in its early progress but between *c* and *b* has given rise to five young

#### EXPLANATORY LEGEND FOR PLATE 9

- A — Stoma (*c*) with sporophytic cells (*a* and *b*) above it, 11-day infection × 640
- B — Longitudinal section of a stoma occupied by receptive hyphae, two of which (*a* and *b*) are multinucleate, 11 day infection × 640
- C — Fertilization at two stomata contributory to one aecium. Multinucleate receptive hyphae (*a*, *b*, and *c*) 11-day infection × 640
- D — Receptive hypha, *a*, with 11 nuclei, leading to stoma near *b* in next section, 11-day infection × 640
- E — Group of fertilized hyphae (*a*, *b*, and *c*) emerging between epidermal cells adjoining an aecium, 11-day infection × 640
- F — Fertilized emergent hyphae (*a* and *b*) between cells of lower epidermis × 640
- G — Emergent hypha (*b*) at upper epidermis with sporophytic cells (*a*, *c*, and *d*) near by, 11 day infection × 640
- H — Dead host cell (*a*) in upper epidermis with sporophytic cells (*b* and *c*) beneath × 640
- I — Sporophytic cell (*a*) growing down between palisade cells from subepidermal region, 11-day infection × 640

branches. The acute angle that the branches make with the parent hypha shows that the direction of growth is away from the stoma, not downward toward it. Again, in Plate 10, C, from an older aecium with denser growth, a diploid hypha can be traced from *e*, not far from a stoma, upward, unbranched at first, then branching freely to right and left between *d* and *a*. It appears, then, that fresh growth from the fertilized stomatal hyphae distributes the new sporophyte generation through the upper area of the growing aecium.

In these cases (pl 10, B, *a* and *c*, and C, *a*, *b*, *c*, *d*, *e*, and *f*) the cells are binucleate. Newly fertilized hyphae may contain from one to many introduced nuclei in addition to the single native nucleus. It is not clear how nuclear pairs could be established so quickly when many nuclei are introduced into a hypha. It may be that in cases like Plate 10, B, C, and E, where the sporophytic cells contain only 2 or 3 nuclei, comparatively few nuclei were introduced at the outset. And in cases like Plate 10, A, where cells with 2 to 9 nuclei are found dispersed throughout the aecium, and Plate 11, A, where nuclear conditions are still more extreme, the original addition of nuclei may have been more abundant.

In the aecium shown in Plate 10, A, there is a beginning of differentiation into areas, vacuoles are appearing in the cells of the lower part of the aecium. This change affects gametophytic and sporophytic cells alike throughout this area.

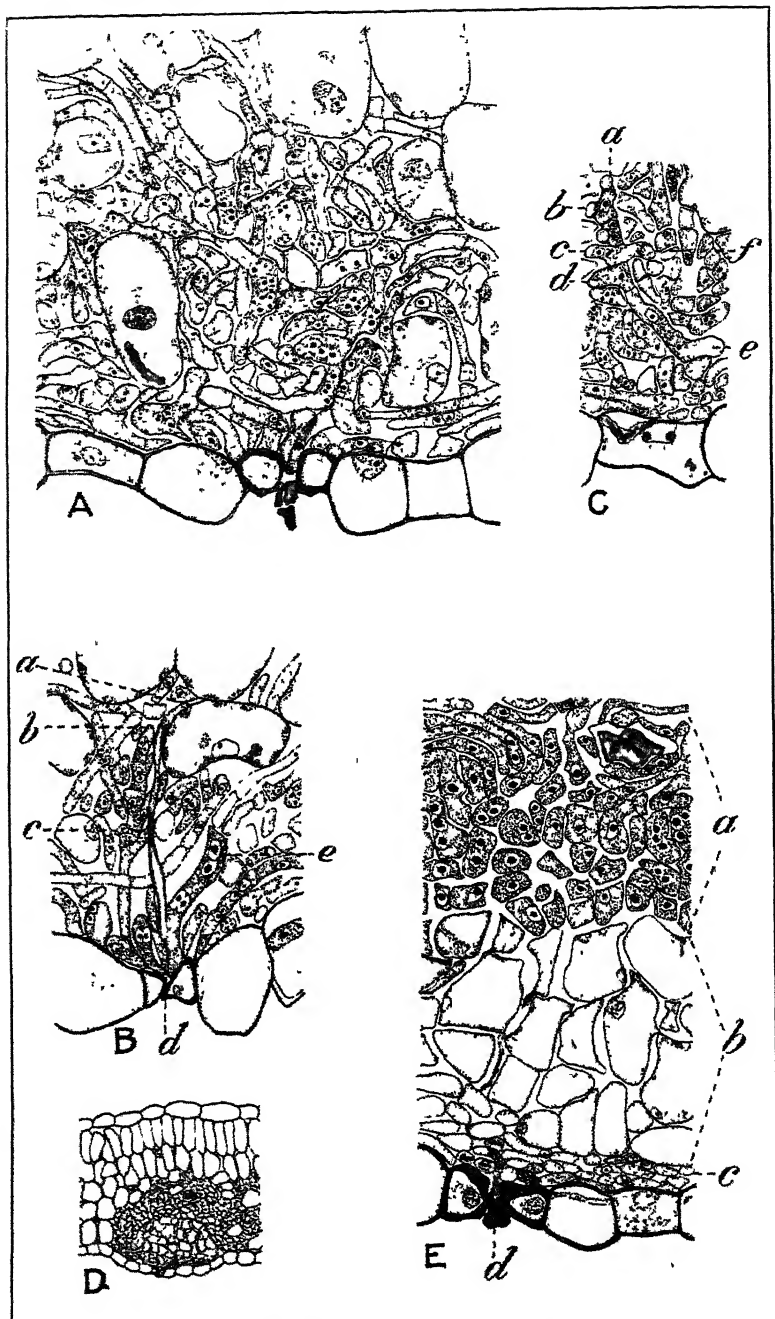
Plate 10, D, represents semidiagrammatically a somewhat older aecium in which this differentiation is well established. There is now an area in the lower part of the aecium, shaped like a thick biconvex lens, composed of large, nearly empty cells that are rapidly dying. This area is capped by a thick layer of smaller, denser cells that will later give rise to the spores. A median strip through this aecium from top to bottom is shown enlarged in Plate 10, E. The contrast in cell size and cell content between the sporogenous area (*a*) and the "space-making" area (*b*) is marked. Remnants of hyphae are still present in the stoma (*d*), but it is no longer possible to trace any connection between them and the upper part of the aecium.

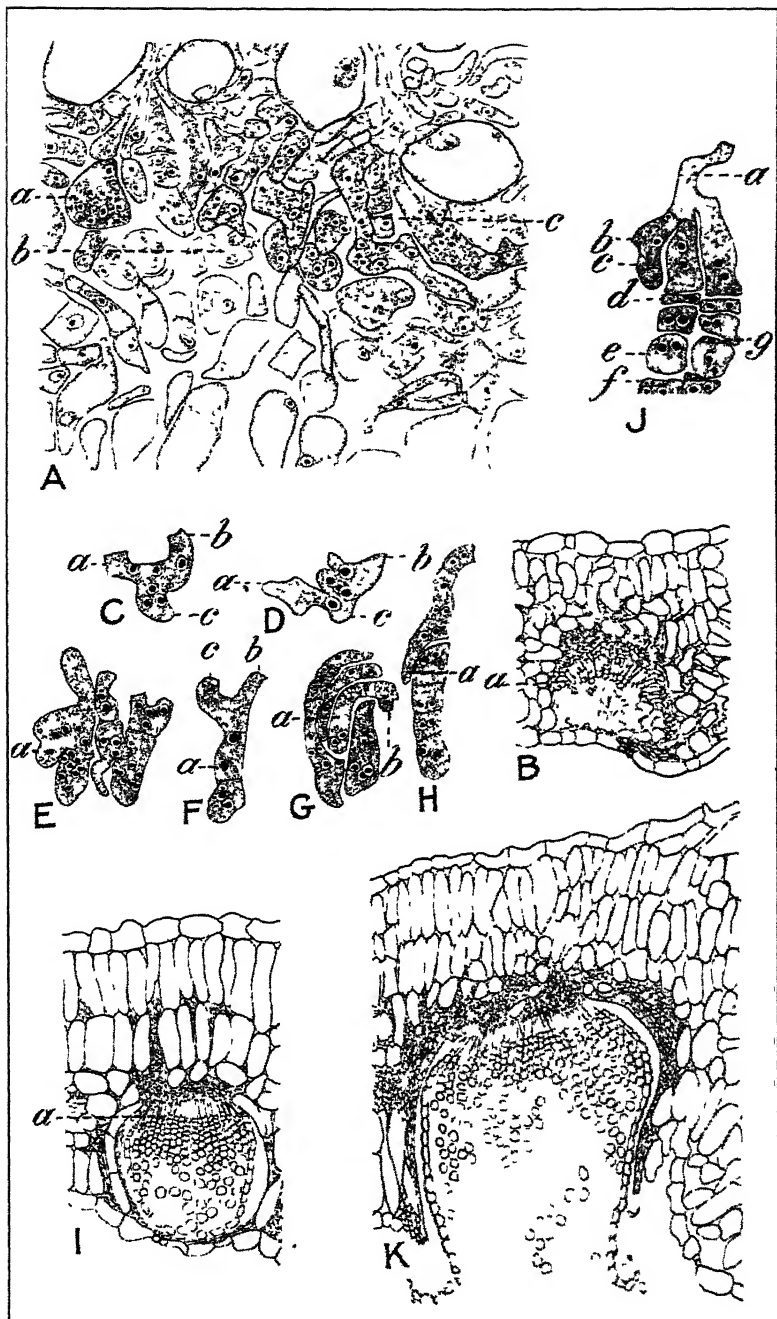
The sporophytic cells (pl 11, A) of the sporogenous area (*a-c*) contain from 2 to 12 nuclei. There are also multinucleate cells in the space-making area (A, *b*), but they are dying. It seems probable that the abundance of nuclei in this case (A) has been continuous since fertilization, especially as younger aecia can be found with the same nuclear condition; but this can not be taken for granted. It is possible that at an intermediate stage rapid growth and cell division reduced the number of nuclei per cell and that later the growth conditions in the sporogenous area favored rapid nuclear divisions, giving rise to the condition found here.

From one cause or another, the average number of nuclei in sporophytic cells of aecia of this age varies widely. Sooner or later, how-

#### EXPLANATORY LEGEND FOR PLATE 10

- A.—Young fertile aecium showing mixture of gametophytic and sporophytic cells. Sporophytic cells with two to nine nuclei, 11-day infection. × 640.  
 B.—Young fertile aecium showing growth of sporophytic hyphae (*e* and *b-c*) from fertilized hyphae at stoma *d*, dead hypha at *a*, 11-day infection. × 640.  
 C.—Portion of young fertile aecium with sporophytic hypha growing from *e*, near stoma, to *a*, with branches (*b*, *c*, *d*, and *f*), 11-day infection. × 640.  
 D.—Diagram of older fertile infection from 11-day infection. × 115.  
 E.—Portion of aecium from D enlarged, showing sporogenous area (*a*), the space-making area (*b*), finer hyphae (*c*), and remnants of receptive hyphae (*d*). × 640.





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ever, the binucleate condition prevails in the sporophytic component of the aecium. This may come while the aecium is still young or may be deferred until spore formation has begun. In the newly fertilized emergent hypha, 1 native nucleus and from 1 to 10 introduced nuclei have been observed. In the final binucleate condition, a nuclear pair is supposed to consist of one male and one female nucleus. Just how the original disparity is changed into the final equality and regularity is not clear. It could be achieved by an unequal rate of nuclear division in the native and introduced nuclei. It may be accomplished by supplementary fusions with haploid cells, although no evidence of this has been seen.

Soon after the stage represented in Plate 11, A, cells of the sporogenous area begin to grow downward into the space-making area to form the initial cells of the spore chains. Plate 11, B, represents an aecium from an 11-day infection in which the layer of basal cells (*a*) is just forming.

Details from the layer of basal cells of this aecium and others of the same stage are shown in Plate 11, C to H. When a cell in the sporogenous area pushes down to assume the function of a basal cell, it may grow from one end or from any point along its length. Plate 11, G, shows several young basal cells, some binucleate and some multinucleate. In three of these the basal cell is merely a continuation of the ordinary apical growth of the hypha. In the fourth (pl 11, G) the hypha is pushing down at two points (*a* and *b*). Plate 11, E, represents another group of young basal cells. The penultimate cell of one (*a*) is pushing out a branch to form a second basal cell. In Plate 11, H, in which a young spore chain is initiated, one of the upper cells of the hypha (*a*) is pushing out a branch. This multiplication of basal cells by branching is common. In Plate 11, J, from an older aecium, the common stem (*a*) has given rise to two main chains of spores, with a younger one starting at *c* and a possible fourth pushing out at *b*.

When a cell extending horizontally pushes down a branch from its center to form a basal cell, the result simulates the 2-legged cell described in rust literature. In Plate 11, D, the irregular cell (*a-b*) has started to form a branch at *c*. In Plate 11, C, the branch (*c*) is somewhat longer. In Plate 11, F, the centrally placed branch (*a*) of the curved cell (*c-b*) has become a fully formed basal cell with one spore mother cell. Figures like this have been interpreted as meaning that two haploid cells (as in pl 11, F, *c* and *b*) fused at their tips, then grew down from the point of fusion to start the diploid spore chain.

#### EXPLANATORY LEGEND FOR PLATE 11

- A — Portion of sporogenous area of aecium from 16-day infection. Multinucleate cells (*a*, *b*, and *c*) × 640  
 B — Diagram of aecium with basal cells forming at *a*, 16-day infection. × 115  
 C — Early stage in formation of basal cell (*c*) from center of curved cell (*a-b*). × 640  
 D — Irregular cell (*a-b*) from sporogenous area. Beginning of basal cell (*c*). Sixteen-day infection. × 640  
 E — Detail from sporogenous layer showing young multinucleate basal cells. Branch forming at *a*. Sixteen-day infection. × 640  
 F — Two-legged basal cell (*a*, *b*, and *c*), 16-day infection. × 640  
 G — Group of young basal cells containing two to six nuclei. Hypha (*a-b*) pushing down at two points. Sixteen-day infection. × 640  
 H — Young spore chain branched at *a*, 16-day infection. × 640  
 I — Diagram of maturing aecium with basal cells at *a*, 16-day infection. × 115  
 J — Detail from aecium in I showing two spore chains from one hypha (*a*), a third spore chain started at *c*, and perhaps a fourth at *b*. Spore mother cells (*d* and *e*) and intercalary cells (*f* and *g*). Sixteen-day infection. × 640  
 K — Diagram of open aecium from 20-day infection. × 115.

Such an interpretation would be obviously incorrect in *Puccinia triticina*, as the sporophyte generation started long before these cells were formed

When first formed, the basal cell may contain from two to eight nuclei (Pl. 11, E and G) An even number of nuclei is usually found, but these are scattered irregularly, not in pairs. The extra nuclei are utilized in supplying nuclei to the first cells of the spore chain, and by the time the chains are well established the basal cells are regularly binucleate (Pl 11, J)

The first cells formed by the basal cells are the spore mother cells Each of these divides into two—a large cell, the definitive spore, and a very small intercalary cell The intercalary cell (pl 11, J, f and g) is seen attached to the spore as an inconspicuous slice from one side

As the parallel, closely packed chains of spores from the basal cells lengthen, they push down into the area of looser dead cells below, crushing them as they grow The peripheral layer of basal cells gives rise to the peridium, a continuous sheath, one cell thick, inclosing the spore mass Plate 11, I, shows a maturing aecium of a 16-day infection, and Plate 11, K, an open aecium from a 20-day infection. The diagrams in Plate 11, B, I, and K, are drawn at the same magnification and show the great increase in the size of the aecium after spore formation sets in, and also the increase in the thickness of the host leaf bearing it.

#### DISCUSSION

The entry of the sporidium into the epidermal cell and the formation of the primary hyphae and its mode of branching are very similar in *Puccinia graminis* (1) and *P. triticina*

Older primary hyphae and older haustoria of *Puccinia triticina* become incased in a layer of material staining like the host cell walls and presumably deposited by the host cell. In the case of some invading parasites, e g, *Ophiobolus graminis* Sacc on wheat roots (11), wall materials are continually built up around the advancing tip of a hypha as it forces its way into a cell, resulting in the finger-shaped "lignituber," through the end of which the hypha may finally escape into the cell lumen In *P. triticina* entrance into a host cell appears to be unimpeded, but later the fungus may become insheathed by wall materials This sheath is probably a partial defense of the host against the intruder

Dodge (10, p 1751) and others "choose to view today the picture of the rust life cycles against the background of a red alga ancestry" The finding of functional receptive hyphae in the rusts adds interest to such comparisons and increases the probability of phylogenetic relationship between the two groups No generalizations can be made, however, until it is known how widespread and how varied this mode of fertilization is among the rusts

In *Puccinia triticina* functional receptive hyphae form at both the upper and the lower surface of the leaf By far the greater number, however, are in the stomata of the lower surface, and it is here that fertilization ordinarily takes place. Fertilization is doubtless aided by the distribution of the spermogonia, at least half of which open upon the lower surface of the leaf. Because of this, visiting insects,



bilities and so affect the percentage of fertile doubles Hartmann (13) and Kniep (14) have summed up a number of more or less similar cases found among the algae and fungi

In hybridizing varieties of *Puccinia graminis*, Stakman, Levine, and Cotter (18) obtained several physiologic forms from the spores of one aecial cup. On the other hand, in crossing physiologic forms, Newton, Johnson, and Brown (16), as a rule, isolated but one physiologic form from one aecium. The occurrence of only one or of more than one physiologic form depends on whether the sporophytic growth in any one aecium is descended from a single pair of conjugate nuclei (or several similar pairs) or from several unlike pairs. In *P. triticina* the mechanism of fertilization is certainly adequate to permit several physiologic forms to enter into the composition of one aecium. With several hyphae emerging at one stoma and several occupied stomata underlying one aecium, and with occasional accessory points of fertilization between epidermal cells of the lower epidermis and a still further possible contribution of fertile hyphae from a fertilization at the upper epidermis, there is ample opportunity for the introduction of spermatial nuclei. Any one of these receptive hyphae may receive from one to a dozen outside nuclei. But while the facilities for fertilization would permit the introduction of several physiologic forms, it would be uncommon in nature for spermatia of several forms to mingle on so small a part of the leaf surface as to enter the same aecium.

The mechanism for the distribution throughout the aecium of the introduced nuclei is not fully worked out. Buller (5) finds that in *Coprinus lagopus* Fr, when two haploid mycelia of opposite sex meet, there is a progressive diploidization of each mycelium by the other, effected by repeated nuclear divisions and migrations. A hypha of one mycelium touches a hypha of the other mycelium, fuses with it, its nucleus moves over, then divides, and one of the daughter nuclei moves on into the next cell. This in turn divides, sending on one of the daughter nuclei into the next cell. In this way both mycelia are rapidly transformed into diploid mycelia, each cell of which has a pair of conjugate nuclei of opposite sex.

In *Puccinia triticina* an indefinite number of nuclei may be introduced into the haploid receptive hypha. Branches from this hypha originate either near the stoma or farther up in the aecium. These branches may be binucleate from the beginning, but are often composed of multinucleate cells, the nuclei of which look alike. After the initial difference in size between spermatial nuclei and aecial nuclei is lost, there is no means of identifying male and female nuclei. The nuclei do not lie in pairs, but are scattered irregularly through the cytoplasm. Eventually, the binucleate condition is achieved by a more rapid rate of division of the native nuclei, by nuclear migrations, or by cell fusions between adjoining cells. These processes have not been seen, but their existence seems probable. From what is known of other fungi with conjugate nuclei it may be concluded that in the aeciospore one nucleus is of male and the other of female descent, but it would not be possible to prove this in *P. triticina*.

In *Puccinia triticina*, sporophytic growth in the aecium is well established before the layer of basal cells is differentiated. As a consequence, basal cells are formed, ordinarily at least, by cell

attracted by the nectar and going from one infection to another, may transfer spermatia to the lower as well as to the upper surface of the leaf.

Aecia, so far as noted, form only in connection with stomatal hyphae at the lower surface of the leaf. No sterile aecia have been found beneath the upper epidermis. Rarely a fertile aecium opens on the upper surface, but since the history of its development is unknown it is uncertain whether it started with a fertilization through the upper epidermis or through stomata of the lower epidermis. Fertilization takes place occasionally at the upper epidermis, but sporophytic hyphae initiated there apparently become effective only by growing downward and becoming part of an aecium below.

The fertilization of emerging hyphae at points some distance from an aecium and the spread of sporophytic mycelium from these points may explain some of the hitherto baffling cases of intermingled sporophytic and gametophytic mycelia in the rusts. Sporophytic hyphae have been found mingled with the mycelium of the gametophyte generation by Blackman and Fraser (4), Olive (17), Lindfors (15), Walker (19), Allen (1, 2), and others.

The word "spermogonium" implies a male structure. It should not be forgotten, however, that when nectar is interchanged between two infections of different sexes each fertilizes the other. Genetically, as Craigie (8) pointed out, the spermogonia of one infection are of one sexual group, while the spermogonia of the other infection are of another group.

The majority of the infections bear both spermogonia and emergent hyphae, so the haploid generation must possess the genetic basis for both structures. The proportion of spermogonia to emergent hyphae, however, varies widely in different cases, some infections bearing many spermogonia and few receptive hyphae, others fewer spermogonia and more receptive hyphae, and a few no spermogonia and many receptive hyphae. Whether this is due to differences in the environment or to genetic differences in the individuals is not certain. The host plants were given similar treatment. Infections of the same age, borne on the same host plant at the same time and resulting from the same inoculation, showed the full range of variation. As great a variation in the proportion of spermogonia to aecia was found in infections on younger leaves as was found on older leaves. If this variation is the result of varying conditions in the host or in the external environment, it is not clear what factors could be responsible for it.

On the other hand, data at present available are not sufficient to prove that the differences are genetic in origin. Of interest in this connection is the fact that an isolated gametophyte, although possessing both male and female organs, remains haploid throughout its life, and that three-fourths of the double infections (instead of the expected one-half) prove fertile. The number of doubles under observation was not great enough to give much weight to this evidence, but it at least suggests that in *Puccinia triticina* more combinations prove fertile than would be expected if the infections could be divided exactly into two equal groups, one (+) and one (-). Further experiments directed toward a genetic analysis of the situation might reveal varying degrees of genetic "maleness" and "femaleness" expressed morphologically in the varying proportions of male and female organs. This might determine the range of mating capa-

during the organization of the sporogenous area. Young basal cells contain from two to eight nuclei. The extra nuclei are utilized in the formation of binucleate spores, and eventually the basal cells become regularly binucleate.

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division and not by fusion. Sometimes a false appearance of fusion is given when a long horizontal cell pushes down a branch at its center to form the basal cell, thus simulating the 2-legged cell described in rust literature. For many years 2-legged cells have been interpreted as meaning that two haploid hyphae meet at their growing tips, fuse at the point of contact, and give rise at this point to a downward-growing binucleate cell, but it is evident that the presence of a 2-legged basal cell is not in itself proof of fusion. In the past the presence of a few 2-legged cells in the layer of basal cells has sometimes been the only evidence offered that the sporophyte originated by fusion of pairs of uninucleate cells in the sporogenous area in the aecium. Without a study of younger stages, there is a risk of error in this assumption.

#### SUMMARY

*Puccinia triticina* Eriks., the leaf rust of wheat, has its gametophyte generation on species of *Thalictrum*. The sporidium, formed by the germinating teliospore in the spring, is a haploid spore, and when it falls upon a *Thalictrum* leaf it germinates, enters directly through the outer epidermal wall, and forms a 4-cell to 6-cell primary hypha in the epidermal cell, which in turn gives rise to haploid intercellular mycelium.

After six or seven days of vegetative growth, reproductive activities set in. Spermatogonia form in about equal numbers at the two surfaces of the leaf. At the same time receptive hyphae grow into stomatal apertures or between epidermal cells of the upper or the lower epidermis. On young tender leaves an extensive growth of mycelium may be present beneath the upper epidermis, giving rise to groups of upright hyphae pushing up between epidermal cells.

*Puccinia triticina* is heterothallic. A monosporidial infection may bear both spermatogonia and receptive hyphae, but if kept isolated it remains haploid. Three-fourths of the double infections (i. e., two in contact) produce open aecia.

A comparative study of infections of the same age shows that some have many spermatogonia and few receptive hyphae, others have comparatively few spermatogonia and more receptive hyphae, and a few have no spermatogonia and abundant receptive hyphae.

In the substomatal air space above a stoma occupied by one or more hyphae, other hyphae grow and branch rapidly, forming a dense little nest of cells, the beginning of an aecium. In the absence of fertilization this haploid aecium grows, undergoes the first differentiation into areas, then slowly deteriorates, and dies without forming spores.

When spermatia from another and different infection are brought to an infected area, spermatial nuclei enter the tips of the receptive hyphae. There may be from 1 to 10 spermatial nuclei in each of these hyphae. Fertilization takes place most frequently at the stomata, but may occur also in hyphae emerging between epidermal cells of the lower and the upper surface of the leaf.

Growth from these fertilized cells permeates the aecium. Cells of sporophytic hyphae may contain 2 nuclei, but usually contain more—sometimes 8 or 10. This multinucleate condition may persist

# RESTORATION OF VIRULENCE OF ATTENUATED CURLY-TOP VIRUS BY PASSAGE THROUGH STELLARIA MEDIA<sup>1</sup>

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## INTRODUCTION

In preliminary reports the writer (6, 8)<sup>3</sup> announced briefly the restoration of the virulence of sugar-beet curly-top virus after it had been attenuated by a very resistant host. What appear to be similar cases of this phenomenon with pathogenic organisms that have become attenuated have been reported frequently in animal pathology. For example, Pasteur, Chamberland, and Roux (9) reported that exposing the anthrax organism to a certain degree of heat so reduced its infective power that it was barely able to kill day-old guinea pigs. However, when the attenuated organism was passed successively through several day-old guinea pigs, it gradually increased in virulence until finally it was able to cause the death of a sheep.

The term "attenuation," as used in this paper with reference to curly-top virus, means the reduction in the ability to infect and in the power to produce severe symptoms. Of the two criteria used in determining attenuation, the mildness of symptoms produced is considered more important than the percentage of infection obtained.

In plant-disease literature there are several examples that appear to be cases of virus attenuation. Johnson (4), by means of heat, succeeded in attenuating the mosaic virus of tobacco to such an extent that it produced only a small percentage of infection and very mild symptoms as contrasted with the effect produced by the untreated virus. He was unable to restore the virulence to this virus by repeated passages through susceptible tobacco plants. Later Johnson and Ogden (5) succeeded in occasionally producing an attenuated condition of tobacco mosaic virus by bubbling air and oxygen through the extract of green mosaic plants. This attenuation of the virus was similar to that produced by heat, remaining stable through several subsequent transfers. These two cases of virus attenuation correspond closely to the kind of attenuation discussed in this paper.

Salaman (10) reports modification of the symptoms of crinkle A, a virus disease of potato, after the virus had passed through *Datura*. These symptoms did not resemble a mild form of the original crinkle A, but rather a mild mosaic. When transferring crinkle A to several varieties of potatoes by grafting he secured crinkle on some varieties but mosaic symptoms or acute streak symptoms on others. While the evidence is inadequate to settle the problem definitely, he suggests the possibility that he was working with a mixture of viruses.

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<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 765.

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out on the *Chenopodium*. The nymphs gave the same results as the adults.

The virulent virus, for comparison, was transmitted to test beets by means of leaf hoppers reared on a severely diseased beet

The beets used in this experiment to test the virulent and attenuated virus were susceptible plants in the fourth true leaf stage, and the leaf hoppers from the two sources, beet and *Chenopodium*, were caged on the young beets for three days. These insects were confined on a true leaf of each beet by means of celluloid cylinder cages. Two leaf hoppers to a plant were used in some cases and 10 leaf hoppers in others. The results obtained are given in Table 1.

TABLE 1—*Results of inoculation of susceptible sugar beets with different numbers of leaf hoppers carrying either the attenuated or the virulent form of curly-top virus*

[Leaf hoppers caged on beets for four days]

Number of insects used and form of virus	Beets inoculated	Beets infected		Type of symptoms
	Number	Number	Per cent	
2 leaf hoppers with attenuated virus.....	22	8	36	Mild
10 leaf hoppers with attenuated virus.....	38	21	55	Do
2 leaf hoppers with virulent virus.....	41	29	71	Severe
10 leaf hoppers with virulent virus.....	29	24	83	Do

This experiment, confirming previous work with *Chenopodium murale*, shows the attenuated nature of the virus obtained from this host plant. It shows also that the symptoms were mild irrespective of whether 2 or 10 leaf hoppers were used. It is to be noted that 10 leaf hoppers bearing the attenuated virus from *Chenopodium* produced 55 per cent infection, while 2 leaf hoppers harboring the virulent virus produced 71 per cent infection, severe symptoms being produced in all cases where the virulent virus was concerned.

In carrying along a stock of the attenuated virus, from 50 to 80 leaf hoppers have been caged on young beets in the fourth to sixth leaf stage and the resulting symptoms have remained mild, on the other hand, 1 leaf hopper harboring virulent virus has produced severe symptoms on a young growing beet if it succeeded in infecting the beet at all.

It is evident from these results that the greater the number of insects used to inoculate the beet the greater is the percentage of infection obtained. It is also apparent that 2 leaf hoppers carrying virulent virus may cause a higher percentage of infection and severer symptoms than 10 insects carrying attenuated virus. Since as great a number as 80 leaf hoppers with the attenuated virus fail to produce severe symptoms on young growing beets, while 1 hopper carrying virulent virus will produce severe symptoms, it is concluded that in experiments of this type the dosage of virus is not a factor controlling the subsequent symptoms produced.

#### RESTORATION STUDIES

The plants used in the experiments in the restoration of virulence were *Chenopodium murale*, *Stellaria media*, and the susceptible sugar beets used as test plants and as checks.

Smith (12), working with the crinkle virus of potatoes, passed it through *Datura stramonium* L and then inoculated potato plants. Instead of modified crinkle symptoms, mild mosaic mottling resulted. In his earlier work on potato mosaic, Smith (11) concluded that the various symptoms appearing on different plants inoculated with the mosaic were due simply to variation in host response. In his later work (12) on crinkle of potatoes he suggested the presence of more than one virus.

Carsner (1, 2) reported the first cases of attenuation of the curly-top virus by resistant hosts, among which was *Chenopodium murale* L, in 1919 and 1925. After this Carsner and Lackey (3) reported the attenuation of the curly-top virus when it was passed through very resistant sugar beets. Repeated transfers through susceptible sugar beets failed to restore the virulence to the attenuated virus. Later Lackey (6, 7) recorded additional evidence of this attenuation reaction of curly-top virus when passed through certain resistant host plants.

The idea was conceived that if a resistant host attenuates the virus an exceedingly susceptible host might restore its virulence. Carsner (1) had reported chickweed, *Stellaria media* (L.) Cyr., as a very susceptible host plant, so it was selected for trial.

It has been recorded in literature that the virulence of some plant viruses has been increased by methods of handling. Smith (11) reported that potato mosaic increased in virulence when it was transmitted to healthy White Burley tobacco by the aphid *Myzus persicae* Sulz. He also found that tobacco ring spot was increased in virulence by progressive inoculation through successive generations of susceptible tobacco. This increase continued only up to a certain point and then the virus showed a tendency to revert to its original virulence. Later Smith (12) found that the crinkle virus of potatoes could be passed through successive generations of tobacco and a severe leaf-drop streak obtained which readily killed some varieties of potatoes. The interveinal mosaic of potatoes when passed through tobacco also came out as a severe leaf-drop streak. Smith (12) suggests that probably more than one virus is involved, and states: "It may be suggested then that passage of tobacco merely liberates in some way the streak virus which was already present, so that it attacks every potato variety inoculated." It is difficult to determine whether one virus was increased in virulence or whether the different hosts suppress one virus and liberate another.

#### DOSAGE EXPERIMENTS

It has been suggested that the reduction in percentage of infection and possibly the variation in type of symptoms produced by the apparently attenuated curly-top virus might be attributed to mere reduction in the quantity of virus involved. The results of the following experiment bear on this problem.

Attenuated virus was obtained by inoculating nettle-leaved goosefoot, *Chenopodium murale*, with 50 to 75 leaf hoppers, *Eutettix tenellus* (Baker), carrying the virulent form of virus (2). After five to six weeks these hoppers were removed. The virus was transferred from the *Chenopodium* to test beets, *Beta vulgaris* L., either by placing non-viruliferous adults on the plant or by using the nymphs that hatched

symptoms indistinguishable in severity from those produced by the original virulent virus, it is evident that the virus, after being attenuated, had been restored to its original virulence

The symptoms produced by the virus from the three sources are shown in Figure 2

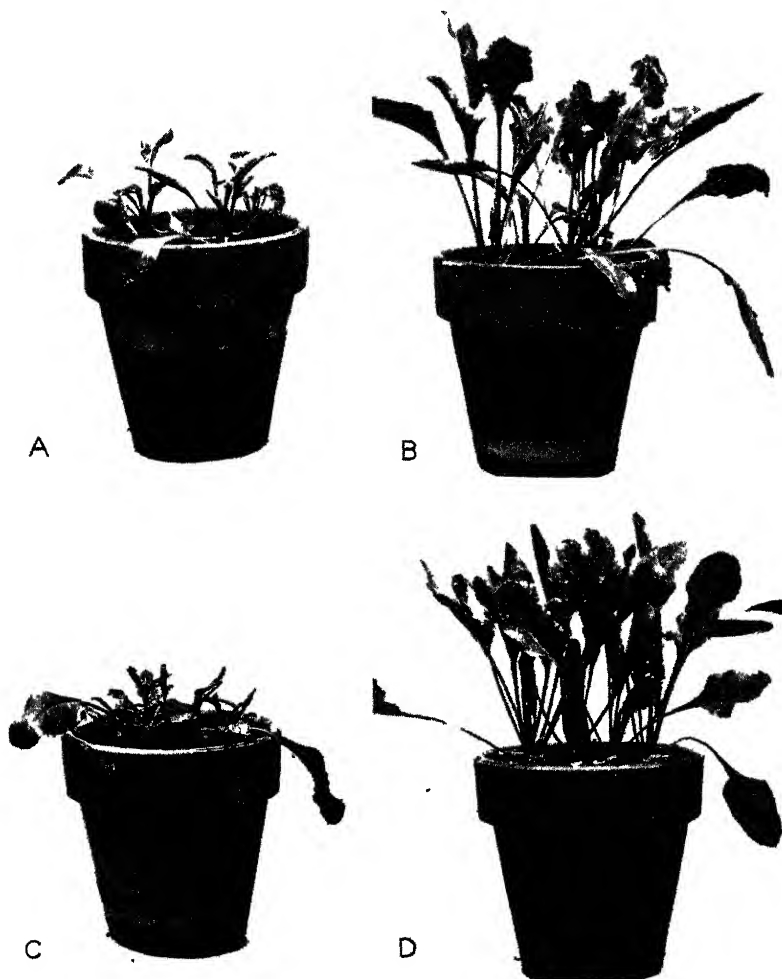


FIGURE 2—A, Beets inoculated with the original virus, B, beets inoculated with the same virus after attenuation by *Chenopodium murale*, C, beets inoculated with this virus restored to its original virulence by passage through *Stellaria media* D, normal uninoculated plants

The test beets used were grown under similar conditions from a seed stock of known high susceptibility, and were all of the same age. The illustration shows the beets seven weeks after inoculation. The beets in pot A were inoculated with the original virulent virus. Those in pot B show the effect of this virus after its attenuation by passage through *Chenopodium murale*. The disease is mild, the

Figure 1, which is a diagram giving data from a typical experiment, illustrates the general plan used in attenuating the virus and restoring its virulence, the original virus being in each case the virulent form of stock virus maintained on susceptible sugar beets

A susceptible beet was used in the beginning to maintain a stock culture of virulent virus. Fifty leaf hoppers from this culture were used to inoculate a *Chenopodium murale* plant<sup>4</sup>. After a period of six weeks, nonviruliferous leaf hoppers were fed on this plant for a week. Four of these hoppers bearing the attenuated virus from the *Chenopodium murale* were used to inoculate a chickweed, *Stellaria media*<sup>5</sup>. This chickweed was a young, fast-growing plant with three stems. The four insects were caged on the plant by means of a small, cylindrical, celluloid cage. The cage was left on for three days. During the incubation period of the disease the chickweed was kept in a semishaded part of the greenhouse under moist and good growing

SOURCE OF VIRUS	CONDITION OF BEET TEST PLANTS
BEET → ↓ CHENOPODIUM → ↓ CHICKWEED →	6 OF 7 SEVERELY DISEASED
	6 OF 33 MILDLY DISEASED
	29 OF 37 SEVERELY DISEASED

FIGURE 1—Plan used in the attenuation and restoration of the curly-top virus, with data from a typical experiment. The arrows indicate the successive passage of the virus through the different host plants, the heavy arrows representing the virulent form of the virus and the light arrows the attenuated condition.

conditions. Six days after inoculation curly-top symptoms became visible. Non-viruliferous leaf hoppers were then caged on the diseased chickweed for three days. These leaf hoppers were then transferred to young healthy beets which were in the 2-leaf and 4-leaf stages, 2 leaf hoppers being used to a plant. In this manner 37 beets were inoculated, 29 of which became infected and showed severe symptoms. At the same time 7 young beets were inoculated with leaf hoppers from the beet on which the stock culture was maintained and 33 beets were inoculated in like manner with virus from the *Chenopodium* plant. Six of the 7 beets (fig. 1) inoculated with the virus directly from the beet (virulent) became infected and showed severe symptoms. These symptoms were indistinguishable from those produced by the virus obtained from the chickweed. On the other hand, of the 33 beets inoculated with virus directly from *Chenopodium*, only 6 became infected and these showed very mild symptoms. The tests with the virus from *Chenopodium* show that the virus used to inoculate the chickweed was attenuated. From the fact that 29 of the 37 test-beet plants infected with this attenuated virus after it has been passed through chickweed had

<sup>4</sup> Plants used in the course of this work were approximately 6 weeks old and 4 to 5 inches high. As Chisner (2) has shown, in some cases no virus can be secured from such inoculated plants, while in other cases only attenuated virus is obtained.

<sup>5</sup> Plants used were approximately 6 weeks old with stems ranging from 1½ to 2 inches in length. It should be noted that passing the attenuated virus through chickweed does not always result in complete restoration of virulence. The critical factors influencing restoration have not been determined.

same age and inoculated on the same date, seven weeks before they were photographed. The extreme shortening of the leaf petioles and general dwarfing of the entire plant, characteristic of the virulent virus, is shown in the plant inoculated with the virus from chickweed.

Several additional experiments were performed. The procedure was in general the same as that used in the preceding experiments. Table 2 gives the results of some of these experiments.



FIGURE 4.—A beet of the same age as the one shown in Figure 3 and inoculated at the same time but with virus that had been attenuated and later restored by passage through *Stellaria media*.

TABLE 2.—Results of inoculations of sugar beets with attenuated and virulent forms of curly-top virus before and after passing through *Stellaria media*.

[Ten leaf hoppers were fed on each test beet for three days]

Virus used	Beets inoculated	Beets infected		Type of symptoms
		Number	Per cent	
Before passage				
Virulent	29	26	89	Severe
Attenuated	30	14	46	Very mild
After passage				
Virulent	10	10	100	Severe
Attenuated	10	10	100	Do

The results given in Table 2 show that the attenuated virus obtained from *Chenopodium murale* before passage through chickweed produced only very mild symptoms on the 14 beets infected. However, after passage through chickweed the symptoms were indistinguishable from those produced by the original virulent virus. The increase in the amount of infection from the virulent form of the virus after passage through chickweed is probably not significant because of the small number of beets used.

Another series of experiments (Table 3) were conducted over a period in which climatic and other environmental conditions varied a great deal, but the results of the various individual tests were practically of the same type and not at all contradictory. In series 1

plants showing only slight vein distortion and curling of the younger inner leaves and no shortening of petioles or dwarfing. In contrast, the plants in pot C show the effect of this virus after its subsequent passage through chickweed. The symptoms, extreme dwarfing accompanied by severe curling of the leaves and severe vein distortion, were indistinguishable from those produced by the direct transfer of the virulent virus (pot A). Normal uninoculated beets are shown for comparison (pot D)



FIGURE 3 —Beet infected with virus attenuated by passage through *Chenopodium murale*

Figure 3 shows in detail the mild symptoms produced by the attenuated form of the virus. Only the younger leaves are affected, the symptoms being slight vein roughening and curling of the blade. The leaf petioles are of normal length, and the entire plant is almost normal in size. This beet is one of the four shown in Figure 2, B.

Figure 4 shows a test beet inoculated with the virus that had passed successively through *Chenopodium murale* and *Stellaria media* and shows in detail the symptoms produced by what is called, in this paper, the restored virus. The beets in Figures 3 and 4 were of the

While there were differences in percentage of infection in the individual experiments comprising these two series (Table 3), the respective average amounts of infection produced by the three forms of the virus and the respective types of symptoms are similar in the two series

Table 4 represents a summary of the inoculations performed in series 1 during September, 1930. The average incubation periods are representative of those found throughout these two series of inoculations

TABLE 4—*Summary of comparative tests performed in September, 1930, with the three forms of the virus*

[Inoculations were made by using two leaf hoppers to each test beet plant]

Kind of virus used	Beets inoculated	Beets infected		Average incubation period	Type of symptoms
	Number	Number	Per cent	Days	
Original (virulent).....	65	42	64	9.5	Severe
Attenuated.....	83	21	25	13.1	Very mild
Restored.....	110	82	74	9.9	Severe

As Table 4 indicates, the average incubation period of the original virulent virus is slightly shorter than that of the restored form, but the difference is probably not significant. By contrast, the incubation period of the attenuated form of the virus averaged considerably longer than for either of the other two forms, and this difference seemed to be consistently maintained. While in this series of inoculations the percentage of infection produced by the restored virus was somewhat greater than that produced by the virulent form, the average for all experiments was slightly less. It is not believed that the differences are significant.

Weighings were made of the entire plants while green, in order to get some measure of the effect of the three forms of virus on the size and development of the infected beets. Table 5 shows the total and average weights of a number of beets infected with virulent, attenuated, and restored forms of the virus, as compared with healthy normal beets of the same age. Forty-two plants were used in each lot.

TABLE 5—*Comparisons of green weights of normal sugar-beet plants and of plants infected with original, attenuated, and restored forms of virus*

[Weighings made eight weeks after inoculation]

Kind of virus used	Beets weighed	Total weight	Average weight
	Number	Grams	Grams
Original (virulent).....	42	138	3.3
Attenuated.....	42	407	9.7
Restored.....	42	153	3.6
Healthy checks.....	42	485	11.5

While the beets infected with the original virulent virus and the restored form are generally severely affected, occasionally among these there is a beet that appears to be somewhat mildly diseased. The

the attenuated virus was obtained from stock cultures on susceptible sugar beets. These cultures were originally secured by transfers from *Chenopodium murale* to beets and were maintained by repeated transfers from beet to beet. Likewise the restored form of the virus which was originally obtained by transfers from *Stellaria media* to beets was in this series obtained from stock cultures maintained on susceptible beets. In series 2 the restored virus was taken directly from chickweed at the date of each inoculation. The attenuated virus was transferred directly from *Chenopodium murale* each time. The virulent virus in each series was obtained directly from beets infected with the stock virus.

TABLE 3—Results of inoculations of sugar-beets with virulent, attenuated, and restored forms of curly-top virus

SERIES 1<sup>a</sup>

Date of inoculation	Virulent form of virus			Attenuated form of virus			Restored form of virus		
	Beets inoculated	Beets infected	Type of symptoms	Beets inoculated	Beets infected	Type of symptoms	Beets inoculated	Beets infected	Type of symptoms
July 14				4	2	Mild.....	4	4	Severe
July 16				4	1	do.....	12	4	Do
July 17				8	6	do.....	12	5	Do
July 22				8	2	do.....	12	10	Do
July 25				6	1	do.....	5	3	Do
July 26				4	3	do.....	4	4	Do
July 29				10	2	do.....	5	4	Do
Aug. 2				11	7	do.....	4	4	Do
Aug. 4				6	3	do.....	6	4	Do
Aug. 11				8	0	do.....	12	12	Do
Aug. 16	4	4	Severe	8	1	Mild.....	7	7	Do
Aug. 18				7	6	do.....	9	9	Do
Aug. 20	14	13	Severe	13	2	do.....	6	6	Do
Sept. 4				8	4	do.....	8	8	Do
Sept. 5	7	2	Severe	6	2	do.....	6	6	Do
Sept. 9	6	4	do.....	13	5	do.....	21	11	Do
Sept. 12	7	6	do.....	33	6	do.....	37	29	Do
Sept. 18	20	14	do.....	20	7	do.....	19	15	Do
Sept. 20	18	16	do.....	15	2	do.....	19	14	Do
Total.....	76	59		192	62		208	159	
Percentage.....		77.6			32.3			76.4	

SERIES 2<sup>b</sup>

Sept. 29	8	5	Severe	8	2	Mild.....	20	16	Severe
Sept. 30				26	1	do.....	30	22	Do
Oct. 2	7	5	Severe	16	8	do.....	38	25	Do
Oct. 3	8	8	do.....				28	14	Do
Oct. 7	7	6	do.....	20	1	Mild.....	27	25	Do
Oct. 11	12	11	do.....	12	1	do.....	26	19	Do
Oct. 15	12	11	do.....	12	0		23	18	Do
Total.....	54	46		94	13		192	139	
Percentage.....		85.2			13.8			72.4	

<sup>a</sup> All virus strains from "stock cultures" carried in sugar beets

<sup>b</sup> Virulent virus from beet "stock cultures," attenuated virus direct from *Chenopodium*, restored virus direct from *Stellaria*

The point to be noted from these two series of inoculations is that the results were very similar whether the restored form of the virus was transferred directly from chickweed to beets or was maintained continuously on beets before being used in these inoculations. This indicates the degree of stability of virus condition obtained.

that the virus involved was as virulent as that in the companion beets that were infected with the same virus and showed the typical severe symptoms

*Stellaria media* is probably of little importance in restoring the virulence of the virus under natural conditions.

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disease is not so mild as that produced by the attenuated virus nor so severe as that usually produced by the restored form. Tests were conducted to determine the condition of the virus in a beet infected with the restored virus which showed such apparent lessening of virulence in comparison with the standard reaction for the same virus. By means of nonviruliferous leaf hoppers the virus was transferred from the typical and atypical plants. Sixteen beets were inoculated with virus from the beet with the milder symptoms, 12 beets were inoculated with virus from the beet with severe symptoms, and 8 beets were inoculated as checks with virulent virus from a stock culture. In the first lot 14 of the 16 beets became infected, in the second, 10 of the 12, and in the third, 6 of the 8 beets became infected. The symptoms in all lots were about equally severe, but none resembled the symptoms of the mildly diseased beet used as a source of virus. Later, virus was again transferred from the two beets used in the test just described, sets of 11 young plants each being inoculated with the virus from each beet. All of these beets became severely diseased, with identical symptoms. These tests indicate that this apparent difference in symptoms may be attributable to an individual host response and is not a contradiction of the general findings.

#### DISCUSSION

While chickweed (*Stellaria media*) is the only plant tested extensively for restoration qualities, it is probably not an important factor in restoring virulence of the virus under natural conditions, because it grows in moist and shady places which are unfavorable to the leaf-hopper vector, *Eutettix tenellus*. This insect prefers dry and warm places in the semiarid regions. Alfalfa (*Erodium cicutarium* L'Her), however, which plays an important rôle in the overwintering of the virus, is a very important host for the leaf hoppers in California during the winter and early spring. Investigations are now in progress to determine its effect on the attenuated virus.

#### SUMMARY

Virulent virus of the sugar-beet curly-top disease has been attenuated by passing it through *Chenopodium murale*. Experimental evidence indicates that this attenuation is due to a change in the quality of the virus rather than to the quantity of virus involved. The attenuated virus remained stable even though passed through successive generations of very susceptible sugar beets.

The attenuated virus has been restored to its original virulence by passing it through *Stellaria media*. Virulent virus passed through *S. media* remained unchanged.

The incubation periods of the virulent and restored forms of the virus were practically of the same length, but the attenuated virus had a much longer incubation period.

The average weight per beet attained by the plants infected with the attenuated virus was almost three times that attained by beets inoculated with either the virulent or the restored forms of the virus.

Tests of the virus from a beet infected with the restored virus which showed somewhat milder symptoms than normal indicated

# VITAMIN CONTENT OF THREE VARIETIES OF SPINACH<sup>1</sup>

By HILDA BLACK KIFER, *Assistant Nutrition Chemist*, and HAZEL E. MUNSELL, *Senior Nutrition Chemist in Charge, Nutrition Studies Section, Bureau of Home Economics, United States Department of Agriculture*

## INTRODUCTION

Spinach (*Spinacia oleracea* L.) is recognized as one of our most valuable greens because it is an excellent source of vitamins and is available in the market all through the year. Since it is so extensively used many varieties have been developed, some of them differing materially in leaf shape, character, and color. Some varieties have smooth leaves, whereas the leaves of others are highly wrinkled or "savoyed." Leaf color varies from light yellowish green to dark green and bluish green.

The Bureau of Home Economics was interested in determining whether a relationship existed between leaf type or color and the content of vitamins A, B, and C. Accordingly, in 1928 a study was undertaken in cooperation with the Maryland Agricultural Experiment Station to determine the vitamin A, B, and C content of several selected varieties of fresh spinach.

For two years preceding this study the Maryland station had been growing different varieties of spinach to determine those best adapted to local conditions of cultivation. All of the spinach used by the Bureau of Home Economics in its tests was grown on the same plot of ground and during the same season.

## REVIEW OF LITERATURE

### VITAMIN A

The association of vitamin A with greenness of plant tissues has been demonstrated by several investigators. Dye, Medlock, and Crist (2)<sup>2</sup> have shown that the vitamin A content of lettuce varies more or less directly with the greenness of the leaves. Kramer, Boehm, and Williams (4) found that the green outer leaves of California head lettuce of the Iceberg variety were thirty or more times richer in vitamin A content than the whitest leaves from the centers of the same heads. Steenbock and Sell (9) made a chlorophyll analysis of etiolated leaves, entirely free from chlorophyll obtained from fresh heads of cabbage plants which at the end of the growing season had failed to head. The analysis showed that the white leaves contained about one-tenth as much pigment as the green leaves, which were found by animal feeding experiments to be superior in vitamin content. Collison and coworkers (1) found that the vitamin A activity of the unsaponifiable fraction from white cabbage was very small as compared with that of the corresponding fraction from the green leaves.

<sup>1</sup> Received for publication Nov. 25, 1931, issued June, 1932. Paper presented before the American Chemical Society, Atlanta, Ga., Apr. 7-11, 1930.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 771.



Figure 1 shows the average gain curves of groups of rats fed 0.025 g, 0.012 g, and 0.006 g portions, respectively, of the three varieties of spinach over a period of eight weeks. Sherman and Munsell have shown that the results obtained are more significant and more consistent when the rate of growth is about 3 g a week. Consequently, the relative vitamin A potencies of the three varieties of spinach are compared by determining the quantity of each which enabled the rats to gain at the rate of approximately 3 g a week. It will be noted that 0.012-g portions of Virginia Savoy, Viroflay, and Princess Juliana gave total gains in weight at the end of eight weeks of 22 g, 24 g, and 22 g, respectively. These results indicate that the three varieties are about equally potent in vitamin A.

This indication is further substantiated by the results obtained from feeding 0.025-g portions of each variety, all of which induced about equal growth rates. Furthermore, the total average gain in weight for the eight weeks of the groups that received 0.006-g portions, respectively, of the three varieties, was in each case 8 g.

#### VITAMIN B DETERMINATIONS

The determination of the vitamin B content of fresh spinach was begun before methods for testing for the two components of vitamin B were available. The method used was that of Sherman and Spohn (8), which makes no distinction between the two factors of vitamin B. All rats were kept in cages having raised screen bottoms and were fed a basal diet of purified casein, 18 per cent; cornstarch, 68 per cent; butterfat, 8 per cent; cod-liver oil, 2 per cent; Osborne and Mendel salt mixture, 4 per cent. In addition to the basal diet the animals were fed graded weighed portions of the three varieties of spinach six times a week.

The average gain curves are given in Figure 2. The groups of rats fed daily portions of 3 g of Virginia Savoy, Viroflay, and Princess Juliana had made total gains in weight of 40.5 g, 39.8 g, and 37.5 g, respectively, at the end of seven weeks (when the test period was terminated). These results indicate that the three samples of spinach contained about equal quantities of vitamin B. This conclusion is further justified by the results obtained when 2-g and 1-g portions were fed. The groups which received 2 g daily of Virginia Savoy, Viroflay, and Princess Juliana had made total gains in weight at the end of eight weeks of 9.5 g, 14.3 g, and 16.3 g, respectively. One gram a day of each sample of spinach just sufficed for body maintenance.

#### VITAMIN C DETERMINATIONS

The method used to determine the vitamin C content of spinach was that described by Sherman, LaMer, and Campbell (6). The basal diet consisted of heated skim-milk powder, 30 per cent; a mixture of equal parts rolled oats and bran, 59 per cent; butterfat, 10 per cent; and table salt, 1 per cent. In addition to the basal diet, a 1-g portion of each variety of spinach was given daily, six times a week. Since the supply of spinach was limited only a small number of animals were used, and the experimental period was terminated at the end of 77 days.

Very little work has been done on the relation of leaf type to vitamin content. A study of the relation of vitamin A content to size of leaves of New Zealand spinach is reported by McLaughlin (5); she found that the vitamin A concentration of the leaf was directly related to its surface area and inversely proportional to its thickness. No other studies on the relation between content of vitamin A and leaf type were found.

#### VITAMIN B

The term "vitamin B" as used in this paper refers to the complex consisting of both the antineuritic and antipellagric factors. No record of a study has been found in regard to the variation of vitamin B content with greenness and leaf form.

#### VITAMIN C

No reference was found in the literature to the relation of antiscorbutic potency to leaf type or color.

### DESCRIPTION OF VARIETIES OF SPINACH (*SPINACIA OLERACEA*)

The following descriptions of the three varieties of spinach which were used in this study are given by Geise and Farley (3): Variety, Virginia Savoy—The plants are vase form, with leaves which are broad, thick, deeply wrinkled or savoyed, and dark green in color. Variety, Princess Juliana—The plants grow very compact, with leaves heavily savoyed and bluish green in color. Variety, Viroflay.—The leaves are smooth or slightly crumpled near the base, spear shaped and thick, and are somewhat yellow green in color.

### EXPERIMENTAL PROCEDURE

#### VITAMIN A DETERMINATIONS

The procedure for the determination of vitamin A was that outlined by Sherman and Munsell (7), with some modification. Young albino rats 28 or 29 days old were placed upon a basal diet devoid of vitamin A. It consisted of purified casein, 18 per cent, cornstarch, 67 per cent; dried brewers' yeast, 10 per cent; Osborne and Mendel salt mixture, 4 per cent; and table salt, 1 per cent. This was irradiated with rays of an ultra-violet lamp at 20 inches for one-half hour. After a preliminary period on this vitamin-A-free diet to deplete the bodily stores of vitamin A, the animals were placed in individual cages and fed graded weighed quantities of spinach six times a week in addition to the basal diet.

A litter of rats was used in such a way that when one rat of the litter was given a daily allotment of Virginia Savoy spinach, two others of the same sex and about the same weight were chosen, one to receive a like amount of Viroflay, and the other a like amount of Princess Juliana. Several litters, comprising a total of 82 animals, were used in this way, and conclusions were drawn from the average of the individual gains in weight. The spinach leaves were picked fresh for each feeding, and the feeding portions of each variety were all taken from similar parts of the leaves. If part of the stem of one variety had to be used, similar amounts of stem sections of the other varieties were used.

TABLE 1 —Results obtained when guinea pigs were fed 1-g portions daily (six times weekly) of three varieties of fresh spinach as the sole source of vitamin C

No	Variety of spinach used to supplement basal diet	Weight of animals during test period			Change in weight of animals during test period	Survival period	Degree of scurvy symptoms at autopsy
		Beginning	Maximum	Final			
98 M.-----	Virginia Savoy-----	Grams 342	Grams 676	Grams 676	Grams +334	Days 77	None
99 M.-----		353	702	702	+349	77	Do
101 M.-----		328	550	550	+222	77	Do
102 M.-----	Viroflay-----	360	806	806	+446	77	Do
103 M.-----		322	366	258	-64	76	None to mild
104 M.-----	Princess Juliana-----	373	690	667	+294	77	Do
105 M.-----		361	388	388	+27	77	Mild
106 M.-----	Control-----	353	692	638	+285	77	Do
107 M.-----		326	326	193	-133	20	Mild to severe

## SUMMARY AND CONCLUSIONS

Studies were made with three varieties of spinach to determine whether any correlation existed between vitamin potency and leaf type or leaf color

The vitamin A content of the three varieties of spinach tested was about equal

The three varieties of spinach seemed equally potent in the vitamin B complex

The results indicate that Princess Juliana probably contained slightly less of vitamin C than the Virginia Savoy and Viroflay

No relationship between leaf type and vitamin A or B content was detected. The variety with heavily savoyed, bluish-green leaves seemed slightly less potent in vitamin C than the other two varieties.

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The results of the feeding tests with the spinach are given in Table 1. The two animals which were fed Virginia Savoy grew well and at autopsy showed no symptoms of scurvy. Of the 3 guinea pigs receiving Viroflay, 2 grew normally, and 1 did not thrive. It died on the seventy-sixth day and at autopsy showed mild symptoms of scurvy.

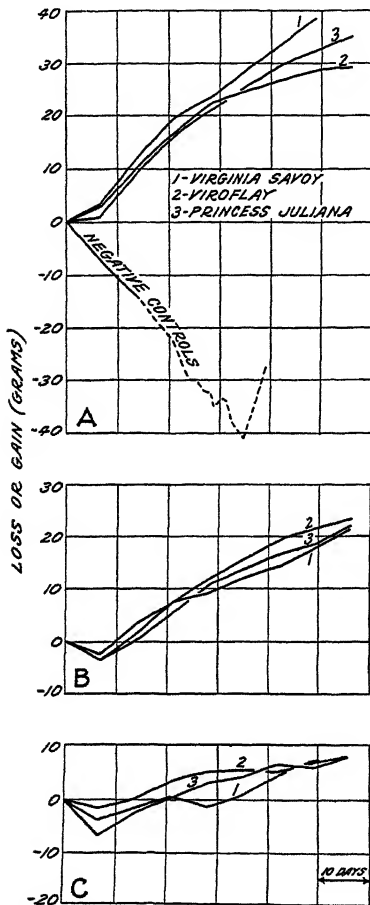


FIGURE 1—Average changes in weight of groups of rats fed 0.025 g (A), 0.012 g (B), and 0.006 g (C) of fresh spinach as the sole source of vitamin A. In the case of the negative controls the broken line represents the average weight of the surviving animals until all had died.

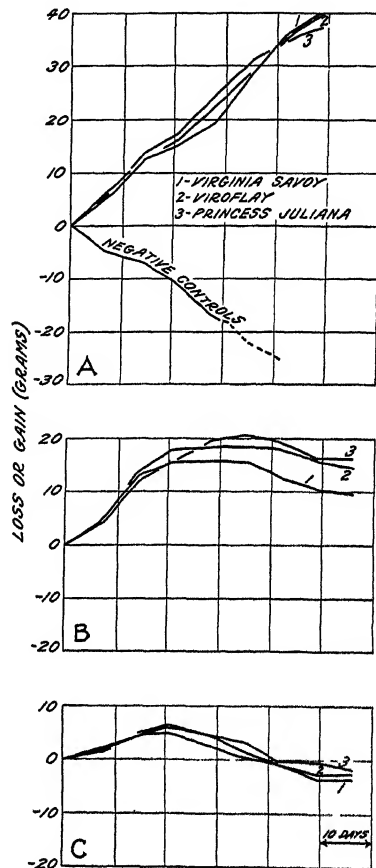


FIGURE 2—Average change in weight of groups of rats fed 3 g (A), 2 g (B), and 1 g (C) of fresh spinach as the sole source of vitamin B complex. The broken line represents the average weight of the surviving animals until all had died.

Three guinea pigs were fed Princess Juliana, and all gained in weight and lived to the end of the experimental period but on autopsy showed symptoms of scurvy. The data are insufficient to warrant definite indications, but it would seem that Princess Juliana was slightly less potent in vitamin C than the other two varieties tested.

# CHEMICAL AND PHYSICAL PROPERTIES OF PETROLEUM SPRAY OILS<sup>1</sup>

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## INTRODUCTION

Petroleum oils have long been employed as insecticides, but the injury that they cause, especially to the foliage of plants, constitutes a serious objection to their use. The literature of the subject has been reviewed by Kelley (8, 9),<sup>2</sup> Green and Johnson (5), and others.

Petroleum oils are highly complex substances. A complete analysis of a crude oil, showing all the individual compounds of which it is composed, has never been reported. The number of compounds in petroleum is very great, and their close similarity is the principal difficulty in separating and identifying them.

The analyses reported in this paper were made for the purpose of determining what properties of an oil may be used as a guide in estimating the injury that it will cause to plants.

## METHODS OF ANALYSIS

For this work, a series of 13 samples of oil were chosen from a large number of spray oils that were available. An effort was made to select samples representative of the oils that were most injurious to plants, those that were least injurious, and a few that were intermediate. The most injurious oils are among those used as sprays on dormant trees and are not generally used on foliage.

The samples may be divided roughly into two classes, the light or highly refined oils, with a sulphonatable portion of less than 16 per cent, and the dark, or poorly refined oils, with a sulphonatable portion of more than 16 per cent. The term refined is used in this work to indicate the degree to which the materials soluble in sulphuric acid are removed, either by sulphuric acid or by liquid sulphur dioxide.

The method used to determine the sulphonatable portion is that recommended by Marshall (12). Twenty cubic centimeters of 37 N sulphuric acid is added in small portions to a 5 c c sample of oil in a Babcock cream-testing bottle, or an American Society of Testing Materials sulphonation bottle, and the bottle is immersed in an ice bath and shaken continuously until the temperature no longer rises. It is then put in a water bath at 100° C and shaken at 10-minute intervals for 1 hour. At the end of that time sulphuric acid, having a specific gravity of 1.84 is added, until the clear unsulphonatable portion rises in the graduated neck of the flask. It is then centrifuged and the portion that has been sulphonated is calculated as the

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<sup>2</sup> Reference is made by number (*italic*) to Literature Cited, p. 786.



was separated from the oily layer of carbon tetrachloride and titrated with standard 0.10 N alkali. The indicator used was a methyl red-methylene blue proposed by Johnson and Green (7). A blank run was made containing all the reagents used in the determination. The difference in the amount of sodium thiosulphate used in the blank and that in the sample represented all the bromine absorbed by the oil.

Viscosity, flash point, and fire point were determined by standard methods used by the United States Government (15) for lubricating oils. Specific gravity, color, and emulsification with water were determined by the methods of Cross (3).

Color was measured against a standard solution containing 5 mg of iodine per 100 c c of water. The readings were taken in a Kleint colorimeter instead of by ordinary comparison tubes as suggested by Cross. The color of the iodine solution was taken as 1 and comparisons were made on this basis.

The values for emulsification are expressed in minutes required for a mixture of 27 c c of oil and 53 c c of water to separate after being stirred according to a strictly specified procedure.

The measurements of surface tension were made with a Du Nouy apparatus. A platinum wire loop exactly 4 cm in circumference was lowered beneath the film to be measured. The force required to pull the loop through the film was determined and expressed in dynes per centimeter of film. The emulsions used for surface film strength measurements were 4 per cent and 8 per cent cresoap, water, and oil emulsions prepared according to the formula for field emulsions of Melander, Spuler, and Green (13). The cresoap was also prepared from fish oil, potassium hydroxide, and cresol by the method of these writers.

Injury to plants was determined by applying the oils to the leaves of barley seedlings. The seedlings were grown in pure quartz sand and a nutrient solution of the following proportions was used:

240 c c of 1 molar $\text{KH}_2\text{PO}_4$
192 c c of 1 molar $\text{MgSO}_4$
192 c c of 1 molar $\text{Ca}(\text{NO}_3)_2$
24 c c of 0.01 molar $\text{FeCl}_3$
23,352 c c of distilled water
<hr/> 24,000 c c total.

For each sample 12 barley kernels were planted in a small jar 10 by 14 cm. Extra jars were always planted so that those which were not up to standard might be discarded. In a light, warm room the seedlings would reach a height of 5 cm in about 7 days. They were then thinned to exactly 10 plants and the oil to be tested was applied carefully on both sides of the leaves with a camel's-hair brush. At the expiration of 3 to 5 days the treated seedlings were cut and weighed. At the same time control seedlings were cut and weighed. The percentage loss in weight of the treated plants, as compared with that of the control plants, that is, the difference in weight between the two, was used as the measure of injury.

Conditions for these experiments can not be exactly standardized, for practical reasons. With a more elaborate equipment of light, temperature, and humidity control, standard conditions might have

percentage of the original sample. With nearly all the samples, the method was found to give results that conformed closely to those furnished by the manufacturers.

Sulphur was determined by the oxygen-bomb method of Cross (3). The sample was completely oxidized in a bomb under 35 to 40 atmospheres pressure of oxygen and the sulphur subsequently determined in the residue as barium sulphate.

The determination of nitrogen was carried out according to the regular Kjeldahl method. A large sample (5.6 g) was taken and digestion was carried out slowly with the addition of successive portions of sulphuric acid until the oil had been completely oxidized and the solution was clear. Considerable care was taken at the beginning of the digestion in order not to cause too much frothing or volatilization of the oil. It was only with the dark, poorly refined oils that any trouble was encountered.

Acidity was measured by titrating a 10-g sample of oil in 50 c.c. of 95 per cent alcohol with 0.10 N alkali. The results were calculated to percentage of oleic acid, as suggested by Cross (3). The hydrogen-ion concentration was determined on the water extract prepared by shaking together for 1 hour 50 c.c. of oil and the same amount of water. A Bailey hydrogen electrode and a standard potentiometer were used to make the measurements.

The method of measuring bromine absorption was essentially that described by Scott (14) and Allen (1), which is a modification of the method proposed by McIlhenny (10, 11). Although bromine numbers are commonly determined on edible oils and drying oils, a satisfactory procedure has in this case been developed for petroleum oils.

Two-gram samples of oil were weighed into glass tubes of a convenient size, made by cutting off small test tubes about 1 cm in diameter. Each tube was then lowered into a clean dry 500 c.c. Erlenmeyer flask and 10 c.c. of carbon tetrachloride was added. Preliminary experiments showed that time and temperature affect the determination, so the reaction was carried out in a bath of melting ice and the time intervals were measured with a stop watch.

A short-stemmed separatory funnel, calibrated to deliver 25 c.c. portions, was fitted into the stopper of an Erlenmeyer flask. The funnel was filled with water, and a cylinder containing 70 c.c. of water and 5 c.c. of 60 per cent potassium iodide solution was set within easy reach.

An excess of approximate 0.33 N bromine-carbon tetrachloride solution was added to the sample in the Erlenmeyer flask. Usually 2 c.c. of the bromine solution was sufficient, but with the darker oils 4 c.c. was required. A large excess of bromine was avoided, as this would have influenced bromine absorption, as would also time and temperature. Immediately after the bromine was added, the separatory funnel, filled with water, was put in place and the stop watch was started. The flask was shaken for exactly 1 minute in the ice bath, after which 25 c.c. of water was added. After another one-half minute of shaking the 70 c.c. of water and 5 c.c. of potassium iodide solution were added. The addition of the last portion of water terminated the absorption of bromine by the oil. The remaining bromine was then titrated with standard 0.10 N sodium thiosulphate, starch being used as an indicator.

The aqueous portion contained the hydrobromic acid formed by bromine and the hydrogen liberated by bromine substitution. This

After developing the barley-seedling method for determining injury it was necessary to show that the results with this method were comparable to those obtained in the orchard. The data given in Table 1 and Figure 1 show the analyses of the oils and the relative injury that they caused to barley seedlings and apple leaves. In Figure 1 the oils are arranged in the order of increasing injury to barley seedlings. Oil No. 24, a colorless, highly refined oil with a paraffin base (the product of an eastern manufacturer) is the least injurious, while oil No. 15, a poorly refined, western oil, with a probable asphaltic base, is the most injurious.

TABLE 1.—Analyses of spray oils and injury produced on barley seedlings and apple leaves

Spray Oil No	Analyses									
	Viscosity (Saybolt at 100° F)	Flash point	Fire point	Color as compared with solution containing 5 mg iodine per 100 cc	Emulsification with water alone (minutes required for oil to separate)	Emulsification with water (cresosol as emulsifying agent)	Surface tension of pure oil	Surface tension of 4 per cent cresosol emulsion	Surface tension of 8 per cent cresosol emulsion	Specific gravity by Westphal balance
		° F.	° F.				Dynes per cm	Dynes per cm	Dynes per cm	
3.....	113	310	345	(1)	0 5	(2)	35 4	40 5	35 4	0 873
4.....	63	280	295	0 20	5 0	(2)	34 6	36 1	35 1	861
5.....	136	315	355	6 00	35 0	(2)	36 6	41 7	41 9	919
8.....	425	360	410	33 33	6 0	(4)	37 0	42 8	39 2	930
12.....	125	315	355	12 50	17 0	(5)	36 0	42 2	41 9	908
13.....	82	300	340	3 60	6 0	(4)	36 3	38 4	38 6	.908
15.....	62	270	300	3 50	7 0	(5)	35 5	40 4	39 7	896
16.....	56	280	310	35	4 0	(4)	34 6	37 1	35 3	.874
20.....	98	330	360	13	5	(2)	34 8	38 1	36 6	.879
21.....	56	260	290	37	3 0	(4)	35 1	37 6	35 3	884
22.....	59	285	305	25		(3)	34 9	37 4	34 9	863
24.....	68	350	390	(1)	5	(2)	34 1	36 3	35 1	.837
28.....	110	325	375	8 50	9 0	(5)	36 1	41 9	39 7	923

Spray oil No	Analyses									Injury to—	
	Sulpho-nat-able portion	Sul-phur	Nitro-gen	Bromo-absorp-tion	Bromo-substi-tution	Bromo-addi-tion	Free fatty acids as percentage of oleic acid	CHX 10 <sup>-4</sup>	pH	Barley seedlings as percentage loss in weight	Apple leaves as percentage loss in weight
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent					
3.....	6 0	0 020	(5)	0 24	0 01	0 22	0 028	0 0136	5 866	14 7	47 8
4.....	12 0	0 021	(5)	89	26	37	0 028	0 0308	5 511	32 1	58 3
5.....	41 6	290	(5)	5 73	2 76	21	170	6420	4 193	52 5	83 8
8.....	53 0	556	0 025	5 94	2 04	1 86	170	0 0320	5 495	38 0	69 2
12.....	27 5	.350	0 030	5 48	2 28	92	0 065	1460	4 835	45 6	70 9
13.....	31 2	.330	0 012	4 93	1 62	1 69	0 028	2080	4 683	59 6	76 3
15.....	26 4	.213	0 011	4 30	1 98	24	.085	5940	4 260	60 7	83 9
16.....	10 0	.110	0 003	.98	26	46	.028	4520	4 345	31 0	83 9
20.....	1 0	.041	.002	.52	.08	36	.028	0 0092	6 036	28 8	40 9
21.....	15 6	.042	.003	1 43	.42	59	.042	0 0455	5 342	40 4	79 6
22.....	2 0	.048	(5)	.24	.06	12	.042	0 0100	6 002	14 4	38 9
24.....	(5)	.042	(5)	.08	.02	.04	.014	0 0116	5 934	7 8	11 0
28.....	35.2	.570	(7)	6 40	3 14	.26	120	1920	4 717	58 7	70 1

1 No color.

2 Very poor

3 Poor

4 Good

5 Very good.

6 None

7 Trace

been more nearly approached. However, the method was quite successful as it was conducted, the results checking closely with those of duplicate determinations run at the same time.

An attempt was made to use bean plants, but because of the lack of uniformity in the plants it was discontinued. A few trials were made with pairs of bean leaves on the same plant, one leaf being treated and the other used as a control. This method is good when the degree of injury is determined by observation alone, but it is not satisfactory when more exact quantitative results are desired.

In order to find out whether or not the effect on barley seedlings would serve at least as an indication of the injury to be expected on apple leaves, a single series of determinations was made in the orchard. Each sample of oil was applied to both sides of 20 leaves on three different limbs, and at the expiration of 16 days the treated leaves were gathered and weighed. This experiment was conducted at a time when the leaves were making very rapid growth. The trial was carried on for too long a period, however, as some of the stronger oils completely killed the leaves, nevertheless the purpose of the experiment was fairly well accomplished.

## EXPERIMENTAL RESULTS

### INJURY

The method of determining oil injury by its effect on barley seedlings was developed after most of the analyses had been made. For a considerable time there has been a need for some standard with

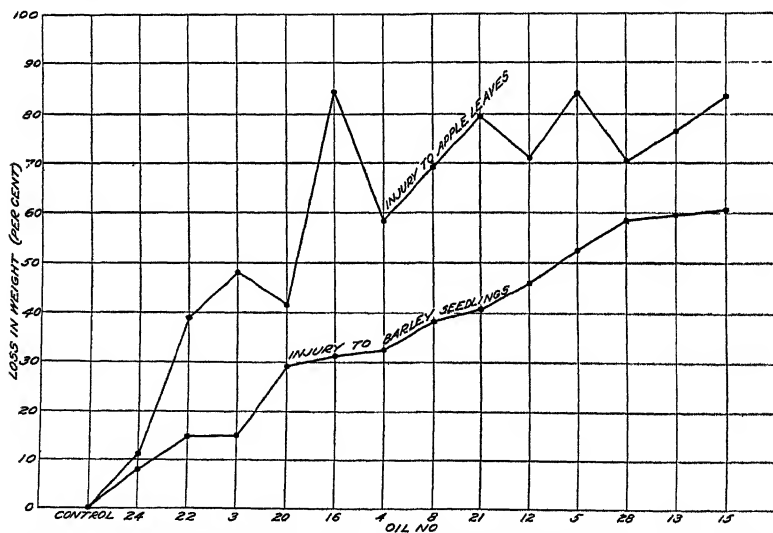


FIGURE 1—Injury done to apple leaves and to barley seedlings when various oils were used for spraying

which the properties of the oils might be compared. Very early in the study Green and Johnson (5) attempted the measurement of oil injury by the changes in the respiration of plants. The changes proved to be too small and uncertain to measure by the methods then available, so the idea was abandoned. However, the results showed that the poorly refined oils caused an increase while the highly refined oils caused a decrease in the rate of respiration of bean leaves.

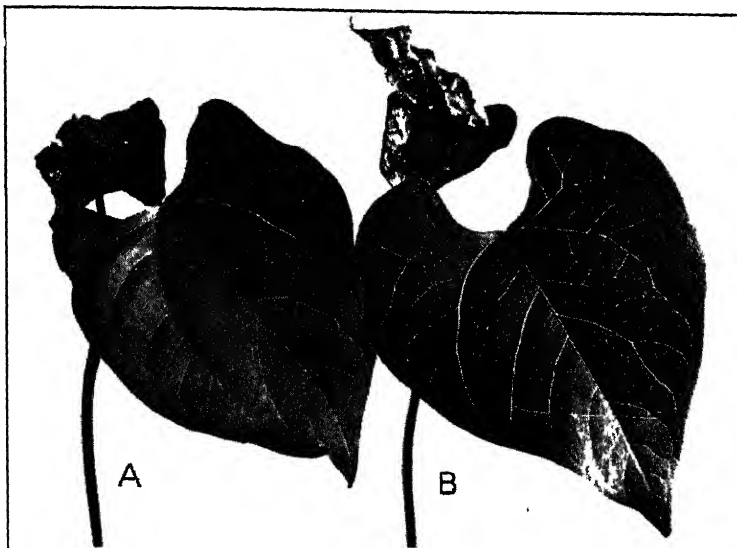


FIGURE 3.—Typical results obtained when dark poorly refined oils were applied to bean plants. A, The badly injured leaf was treated with oil No. 5, which had a sulphonatable portion of 46.1 per cent, B, the badly damaged leaf was treated with oil No. 23 which has a sulphonatable portion of 35.2 per cent.

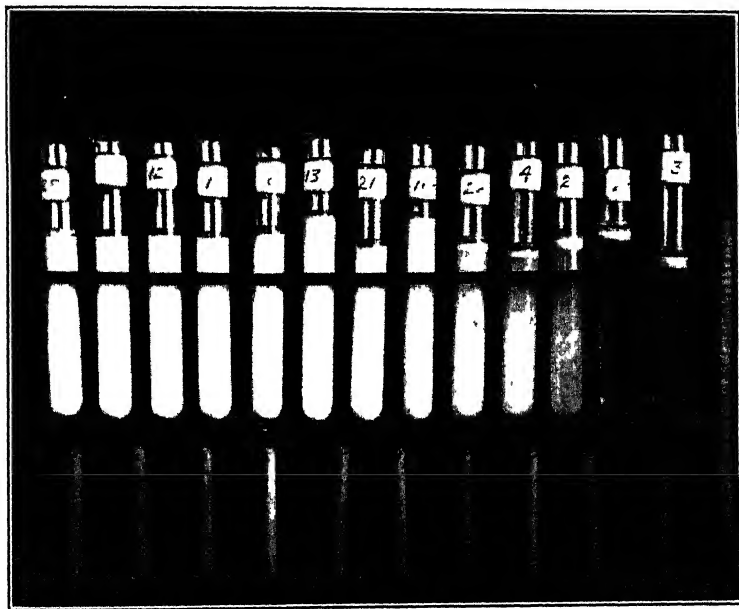


FIGURE 4.—A series of 8 per cent oil-water emulsions, emulsified with cresoap, arranged in the approximate order of increasing stability of emulsions.

The barley-seedling injury curve is the average of three determinations carried on at different times, while the curve for apple-leaf injury is the result of a single series of determinations, and hence is not so regular. With the exception of oil No 16, the results are fairly comparable and indicate that the injury to barley seedlings is a fair measure of the injury to be expected on apple leaves.

As previously stated, some preliminary experiments were made on bean plants. Figure 2 shows two bean plants on which one of each of the pairs of leaves was treated with highly refined white oils, 3 and 24. The treated leaves appear little injured. Figure 3 shows leaves similarly treated with oils 5 and 28. Both of these oils are dark and poorly refined, and they caused very great injury.

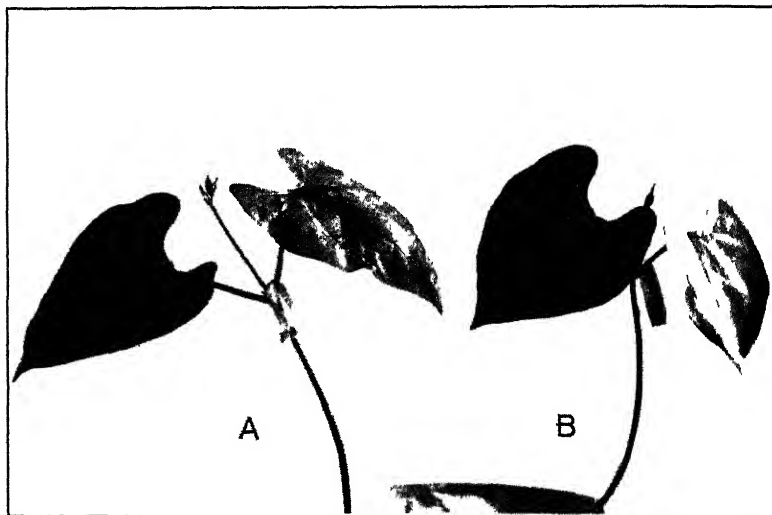


FIGURE 2—Typical results obtained when white oils were applied to bean plants. A, Leaf with collar on its stem was treated with a highly refined oil, No 24, B, leaf with collar on its stem was treated with a highly refined oil, No 3.

#### EMULSIFICATION

It is generally believed that the more easily an oil emulsifies the greater will be its injury to plants. Figure 4 shows a series of 8 per cent cresoap emulsions of the oils arranged in the approximate order of their ability to remain emulsified. These emulsions were all prepared in the same manner and were the samples used in the surface tension measurements. The more transparent tubes are the poorer emulsions and were made from oils 3, 24, 20, 4, and 22. They are among the less injurious. The tubes of more stable emulsions (on the left of fig. 4) are the less transparent, and were made from the injurious oils 28, 5, 12, and 15.

A more quantitative expression of the ease of emulsification of the oils is given in Table 1. Figure 5 shows that the oils which produce the most stable emulsions cause the greatest injury.

#### SURFACE FILM

In attempting to devise a method for the measurement of the covering capacity of the oils it was found that only those oils with a sulphonatable portion of more than 6 per cent would, of their

tension to be found in oils with greater amounts of sulphonatable materials, or those which cause greater injury. The surface tension of the emulsions parallels closely that of the pure oils, as would be expected.

The results of this investigation indicate that the color of an oil is related to the injury that the oil may cause to plants. The relation of color to injury parallels closely the relation of the sulphonatable portion to injury, and it is not known which of these variables is responsible for the damage to plants. Sulphonation, or treatment

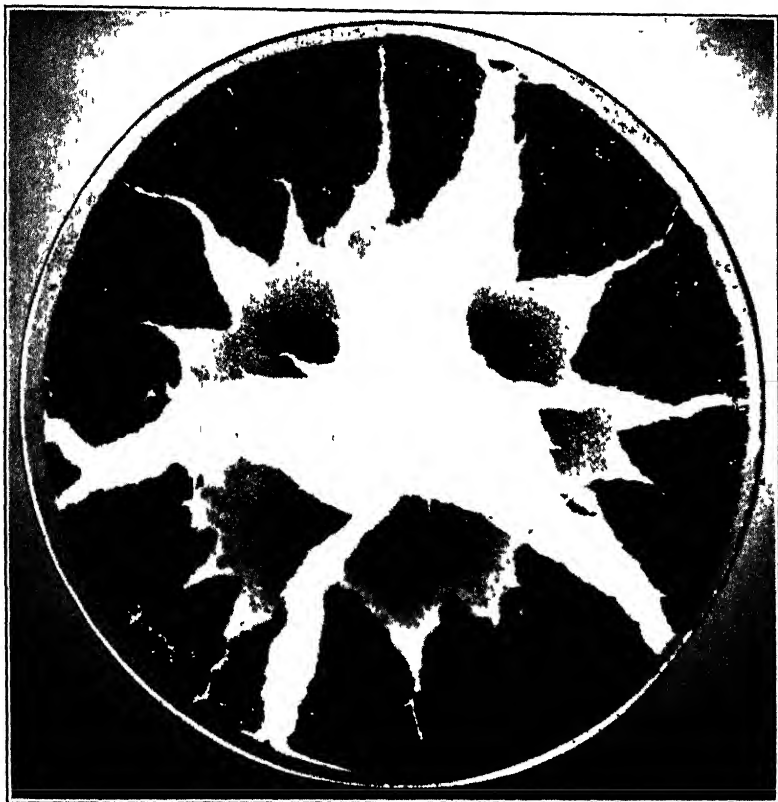


FIGURE 6.—Photograph demonstrating the ability of one oil film to interpose itself upon another. The black outer ring is carbon black dispersed by the first film. The lighter colored particles, covering largely the central portion, were spread upon the first film and were dispersed by the addition of more oil to the center of the surface.

with sulphuric acid, removes the dark materials and, as Bell (2) has pointed out, they may also be removed by filtration, but no data are available on the effect of filtration.

Viscosity, flash point, fire point, and density seem to have little relation to the amount of injury caused by the oils. Viscosity receives a great deal of attention in spray practice, but, on the basis of the data presented here, it does not appear to be related to injury. The values for density show a slight general increase with increasing injury.

own accord, form a film on water. Oils 3, 20, 22, and 24, all with a sulphonatable portion of 6 per cent or less, would not spread on water, and these are among the least injurious. The oils with more than 6 per cent sulphonatable material spread on water, and these are generally more injurious. There is evidently a close relation between the degree of sulphonation and the ability of the oil to spread and also to emulsify. According to the Langmuir theory, the sulphonatable materials, or the materials which spread easily on water, are polar compounds, one portion of which is strongly attracted by water, while the opposite portion is repelled.

The attempts to measure the covering capacity of the oils were made with the object of finally determining the molecular size. After one film of oil had been applied to the surface of the water, the addition of more oil caused it to be driven into a smaller space with a piling up of the molecule to an unknown thickness. Figure

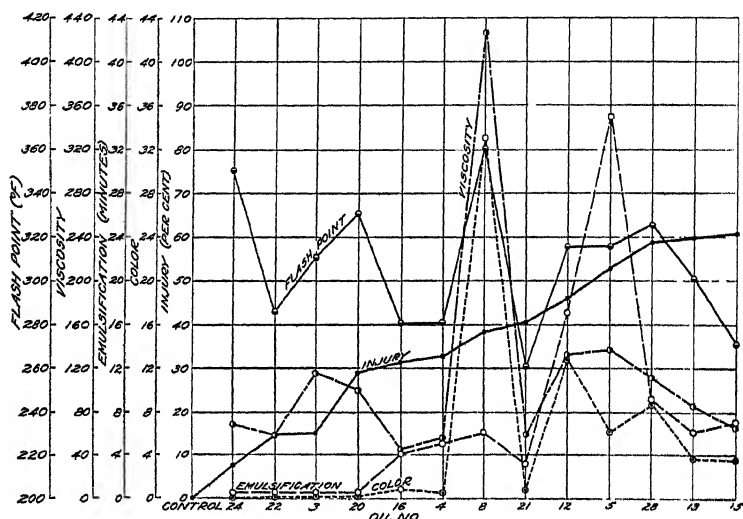


FIGURE 5—Relation between physical properties of oils and their injurious effects upon barley seedlings when used as spray oils

6 shows the action of one film in driving aside another which had already been spread over the surface. The very dark portion around the outer edge of the watch glass, which is carbon black, was dispersed from the surface of the water by the spreading of the first film. The surface was then covered with a lighter colored dust (talc) and this was dispersed by applying more oil to the center of the surface. The process can be continued until the fourth or fifth film has driven the others to the outside. From these results it is apparent that the molecules of oil must be crowded until they are piled up as a polymolecular layer, the thickness of which is irregular and can not be measured.

#### SURFACE TENSION, COLOR, VISCOSITY, FLASH POINT, FIRE POINT, AND DENSITY

As may be seen in Table 1, the differences in surface tension are small. The pure oils vary from 34.1 dynes for oil No. 24 to 37.0 dynes for oil No. 8. If there is any trend, it is for higher surface

Every atomic weight of hydrogen displaced forms a molecular weight of hydrobromic acid. By titration of the hydrobromic acid it is thus possible to calculate the amount of bromine used in substitution. The compounds that take part in substitution are of the unstable class and may be active in plant injury.

The total bromine absorption, minus the bromine used for substitution, is expressed as bromine addition. If the unsaturated compounds of petroleum are ever proved to be the cause of plant injury, the measurement of bromine addition in spray oils will become an important phase of the analysis.

#### SULPHUR AND NITROGEN

Sulphur and nitrogen were present in comparatively small amounts in the oils examined. The maximum quantity of sulphur, 0.57 per cent, was found in oil No. 28 and the maximum quantity of nitrogen in oil No. 12. The fact that some of the very injurious oils, 5 and 28, contain only a trace of nitrogen, or none at all, tends to remove any suspicion that might be held regarding the injurious effects of nitrog-

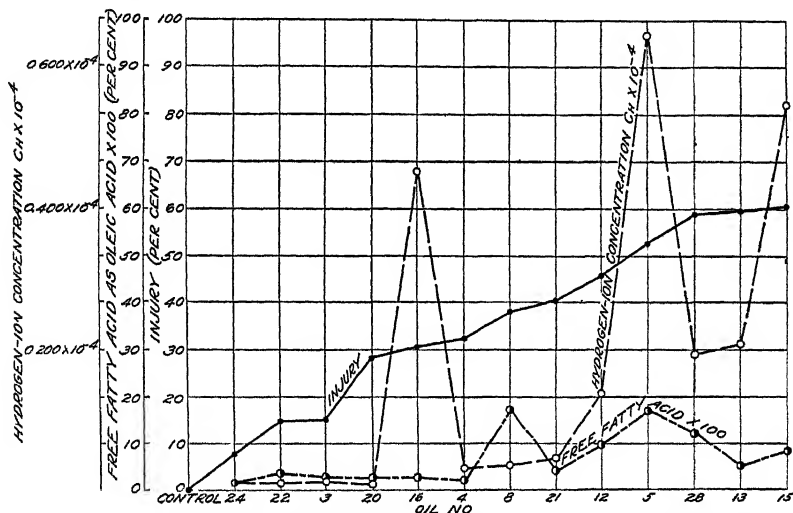


FIGURE 8—The relation of the acidic properties of spray oils to injury of barley seedlings

enous compounds. The larger quantities of sulphur in the oils leave their part in plant injury still an open question.

#### ACIDITY

After the free acids had been run and the slight relation of these to injury noted (fig. 8), the hydrogen-ion concentration was determined. The four least injurious oils, 24, 22, 3, and 20, were found to have a very low hydrogen-ion concentration. From this point on the injury curve the acidity increased irregularly. Oils 16, 5, and 15, have an abnormally high hydrogen-ion concentration.

#### MODIFIED OILS

Because of some of the findings previously mentioned, experiments were conducted to improve the oils, and the search for the portion of the oil causing injury was continued. Since emulsification with

## SULPHONATION

The compounds removed from petroleum oils by sulphonation have been only approximately determined. It is obvious that the more reactive substances would unite with sulphuric acid. Gruse (6, p 121), quotes ZALOZIECKI as giving the following outline for the reaction of sulphuric acid and petroleum.

Sulfur compounds, resins and petroleum acids are dissolved or precipitated. Nitrogen bases and some unsaturated hydrocarbons are combined with the acid. Unsaturated hydrocarbons are polymerized. Some unstable hydrocarbons are oxidized. Aromatic hydrocarbons are sulfonated.

Undoubtedly sulphonation removes materials which cause injury to plants. Gray and De Ong (4) state that the sulphonation test is the best single criterion by which to determine whether or not a spray oil has objectionable properties. Their conclusions are confirmed by the results of this investigation. The greatest inconsistency in the correlation of injury with sulphonation is shown by oils

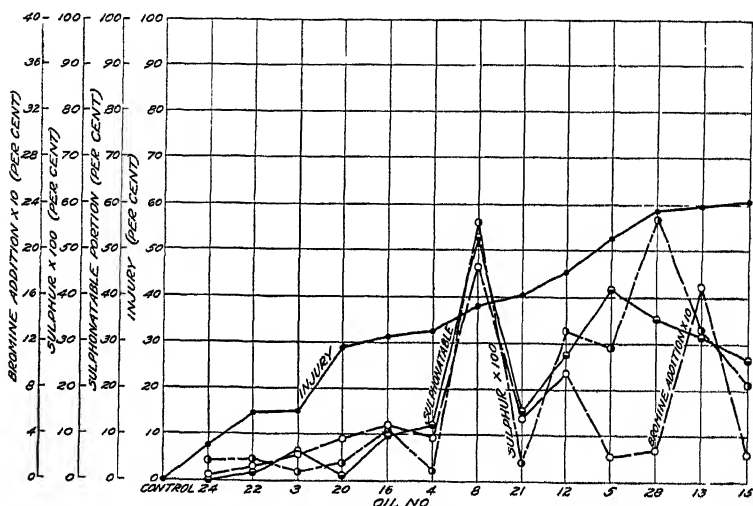


FIGURE 7.—The relation of chemical properties of spray oils to injury of barley seedlings

13 and 15. These are the most injurious oils, but their sulphonatable portion is only 31.2 and 26.4 per cent, which is not so great as oils 5, 8, and 28.

## BROMINE ABSORPTION

As sulphuric acid combines with the more reactive materials, bromine should do somewhat the same. From Figure 7, which presents the values for sulphonation and bromination, it is apparent that there is a relation between the two reactions. Bromine addition is a measure of the unsaturated, or multiple bonded, compounds. Bromine, being very active, easily combines at the point of multiple bonds and thus the compound becomes saturated. In other compounds where hydrogen atoms are held loosely, bromine easily takes the place of these poorly attached atoms and becomes fixed in the molecule by the process of substitution. The hydrogen liberated then unites with uncombined bromine, forming hydrobromic acid,

A series of experiments designed to change the acidity of an oil was made. Oil No 15 was treated in three different ways. The first process was a distillation with steam for six hours in a strong sodium hydroxide solution followed by a thorough washing with water. The second process was a distillation with steam without alkali, and the third consisted in shaking a sample of the oil with an equal quantity of 50 per cent sodium hydroxide in a shaking machine. The treatments with alkali were intended to neutralize any acids present and the steam distillation without alkali would have driven off any easily volatile acids. The relative toxicity of the oils treated by the three processes was determined by their effect on barley seedlings, but no improvement in the oils was observed as the following data show:

*Treatment of oil—Loss in weight of barley seedlings in five days (duplicate determinations)*

	Per cent
Oil, with addition of an equal quantity of 50 per cent NaOH steam distilled for six hours, then washed thoroughly with water.....	65 8
Oil steam distilled and not washed.....	72 7
Oil, with addition of an equal quantity of 50 per cent NaOH, in shaker for five hours, then washed thoroughly with water.....	52 2

Another series of trials was made with oil No. 15. Samples were treated separately with bromine, potassium permanganate, distilled with steam and sulphuric acid, shaken with soil, and the sample that was distilled with steam and alkali in the foregoing trials was washed with a large quantity of very dilute hydrochloric acid and then with water. The following results were obtained.

*Treatment of oil—Loss in weight of barley seedlings in three days (duplicate determinations)*

	Per cent
Shaken with $\text{KMnO}_4$ , washed thoroughly with water, treated with potassium oxalate solution, and again washed thoroughly with water.....	20 3
Shaken with excess of bromine, let stand overnight, and washed thoroughly with water.....	29 7
Oil that was steam distilled with strong alkali (see tabulation above), washed with large quantity of 0.10 N HCl, and then washed thoroughly with water.....	30 7
Distilled with steam and three times its volume of commercial $\text{H}_2\text{SO}_4$ .....	12 5
Shaken with an equal weight of clay soil, centrifuged, and washed.....	21 1
Normal oil, no treatment.....	19 5

The treatment with bromine should bring about complete saturation of the oil, while the action with potassium permanganate, although less definite, should tend to make the reactive compounds in the oil more stable. The distillation with steam and commercial sulphuric acid was only a modified sulphonation, and it will be noted that this was the only one of the processes that produced beneficial results.

The steam-alkali distilled sample brought forward from the work presented in first tabulation above was washed with dilute acid for the purpose of neutralizing any sodium salts of organic acids that might have been formed by the alkali treatment. The experiment was not successful however, for this sample was the most injurious of the group.

The purpose of shaking one sample with soil was to absorb some of the injurious fractions of the oil, but no significant result was obtained.

water had been shown to be related to injury, a small sample of the most injurious oil, No 15, was subjected to long-continued washing with water. A 50 cc sample was put in a small percolating cylinder with an inverted siphon connected with the bottom. Water was allowed to drop into the cylinder, down through the floating layer of oil and out through the siphon. The falling water continually agitated the oil and carried out with it any soluble materials or compounds easily emulsified. The washing was continued for about eight hours and the oil was later tested on barley seedlings. As shown in Table 2, the normal oil caused an injury of 40.6 per cent, while the washed oil caused an even greater injury, 46.1 per cent. Tests were also made to determine the effect of complete sulphonation. Oils 5 and 15 were completely sulphonated according to the standard method. The improvement in oil No 15 was very slight. An injury of 37.0 per cent was caused by the completely sulphonated oil No 15 as compared with 40.6 per cent for the normal, while with oil No 5 the injury decreased from 44.6 per cent for the normal to 24.6 per cent for the sulphonated. In the same series the completely sulphonated oil No 24 caused an injury of only 12.8 per cent. These results indicate that sulphonation of spray oils is not a complete remedy for plant injury.

Oil No. 24 has been previously mentioned as an eastern oil with a paraffin base. Oil No. 3 is probably in the same class, but the other oils are from western fields. In view of the great difference in the degree of injury caused by completely sulphonated oils No 24 and No 15 it is probable that the location from which the crude oil was taken may have had an important bearing on the results. At least all injury can not be attributed to the sulphonatable portion.

TABLE 2—Loss in weight of barley seedlings during an interval of five days after spraying with oils Nos 24, 15, and 5 which had previously undergone various treatments

Oil No.	Treatment of oil	Loss in weight of barley seedlings (duplicate determinations)
		Per cent
24	Normal.....	12.8
	With 1.0 per cent ethyl mercaptan added.....	14.8
	With 0.05 per cent ethyl mercaptan added.....	24.9
	With 1.0 per cent ethyl sulphide added.....	15.2
	With 0.05 per cent ethyl sulphide added.....	19.7
	With 1.0 per cent amylene added.....	16.1
15	Normal.....	40.6
	Completely sulphonated.....	37.0
	Washed for eight hours with water.....	46.1
5	Normal.....	44.6
	Completely sulphonated.....	24.6

The data in Table 2 show the effect of adding two of the probable sulphur compounds of petroleum (ethyl sulphide and ethyl mercaptan) and one unsaturated hydrocarbon (amylene) to oil No 24. In every case the injury was greater than that produced by the normal oil, but there is an apparent inconsistency with the sulphur compounds, in that the greater additions of sulphur caused the smallest increase in injury.

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The data given in Table 2 and in the tabulations on p 785 are from three tests with barley seedlings made at different seasons of the year and they are not therefore comparable. It is obvious that conditions of growth in tests made at different seasons will vary and that comparisons should be made only between samples that are grown side by side and at the same time.

### SUMMARY

A laboratory method for determining the relative injury to barley seedlings caused by different oils and by oils treated by different processes, has been developed. The relation of the injury produced by these oils on barley seedlings in the laboratory to that produced on apple leaves in field experiments has been indicated.

Analyses of different spray oils have been made and the effect of the oils on barley seedlings is shown.

Although no single analysis of spray oils has been found to be directly correlated with injury, the relation of certain properties of the oils to injury is shown. Sulphonatable portion, sulphur, bromine absorption, acidity, ability to emulsify, and color all increase with increasing injury.

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# BULK AS A FACTOR IN FORMULATING GRAIN MIXTURES FOR DAIRY CATTLE<sup>1</sup>

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## INTRODUCTION

For many years treatises on the feeding of dairy cattle have stated that bulk is a factor that must be considered in making up a grain mixture for dairy cattle. A typical recommendation<sup>2</sup> reads as follows:

Heavy feeds, such as cottonseed meal, ground corn, and feeds of that nature, if fed without being mixed with some bulkier feed tend to form a doughlike mass in the stomach when they become moistened and are not easily penetrated by the digestive juices. If, however, the grain ration contains a liberal portion of some bulky feed, as bran or feeds of that nature, it will remain porous when becoming moist in the stomach and will be easily digested. It is very important then that if a cow is being fed a large grain ration, that a liberal portion be of a bulky nature.

Bran is probably one of the most common feeds used to give bulk to the grain ration. In addition it is a feed of high-protein content. Ground corn and cob meal is also often used in the dairy ration. The ground cob, while very low in feed value, has the distinctive value of giving the grain ration bulk. The fiber portions of ground grain sorghum heads are found to perform much the same purpose in that they add bulk.

This recommendation that the grain mixture should be bulky is not the result of experimental investigation. It is the result rather of the line of reasoning that if the grain mixture remains lumped together in the digestive tract, the digestive juices can not act upon it. However, account has not been taken of the fact that the rumen of the bovine is in a state of constant activity, and as a result the lumps or "bol" may be very effectively broken up and their contents mixed with the roughage part of the ration consumed by the animal. It is also possible that some of the bol are regurgitated and broken up by mastication.

Armsby<sup>3</sup> states:

The rumen is so large that it always contains a considerable amount of material and the new feed when swallowed is more or less completely mixed with that already in the rumen by the peristaltic action of the latter, thus tending to prolong its stay. The liquid or comminuted portions probably pass on directly to the omasum, or manifolds, and the abomasum, but the bulk of the feed undergoes the process of rumination.

Colin<sup>4</sup>, by means of a rumen fistula, observed the activity of the rumen and its ability to macerate and mix the newly arrived contents of the rumen and reticulum. He also expressed the opinion that finely pulped material may in part pass directly to the four reservoirs, or that that which does not pass immediately into the last two is soon washed there.

<sup>1</sup> Received for publication Sept. 25, 1931, issued June, 1932. Journal Article No. 92 (new series) of the Michigan Agricultural Experiment Station.

<sup>2</sup> ANDERSON, E. E. FEEDING DAIRY COWS. N. Mex. Agr. Col. Ext. Circ. 94, p. 8, illus. 1927.

<sup>3</sup> ARMSBY, H. P. THE NUTRITION OF FARM ANIMALS. p. 82. New York, 1917.

<sup>4</sup> COLIN, G. TRAITE DE PHYSIOLOGIE COMPAREE DES ANIMAUX, CONSIDEREES DANS SES RAPPORTS AVEC LES SCIENCES NATURELLES, LA MEDICINE, LA ZOOTECHNIE ET L'ECONOMIE RURALE. Ed. 3, enl., t. 1, p. 691-699. Paris, 1886.



The purpose of the present investigation was (1) to determine the ability of the rumen to break up and dissolve such lumps or boli as are formed during deglutition of a grain mixture, and (2) to find whether ground grains pass more or less quickly to the omasum and abomasum or whether they remain in the rumen and are mixed with its contents.

#### EXPERIMENTAL PROCEDURE

In this investigation 32 animals were used, one of which had a rumen fistula. The animals were given their regular feed of grain, silage, and hay about 6 a. m. About noon they were fed 5 pounds of a certain concentrate a certain number of hours before they were to be slaughtered, the purpose being to see how many boli could be recovered after certain feeds were given. Some of the animals were fed the concentrates before they were trucked to a slaughtering house, a distance of about 3 miles, and some were fed afterwards. Some were slaughtered locally and had to be led only about 100 yards. No animals were used that did not clean up the feed offered them within 15 minutes after it was fed. After an animal was slaughtered, the contents of the rumen were carefully sorted over by hand for lumps or boli present.

Linseed meal was used in a large number of these trials, since it is one of the most cohesive of cattle feeds when it becomes moistened. Five animals were fed 5 pounds of ground oats each and slaughtered one hour later. Five were fed 5 pounds of ground corn each and slaughtered one hour later. Three were fed a mixture of  $2\frac{1}{2}$  pounds of linseed meal and  $2\frac{1}{2}$  pounds of ground oats each and slaughtered one hour later. Three were fed  $2\frac{1}{2}$  pounds of linseed meal and  $2\frac{1}{2}$  pounds of ground corn each and slaughtered one hour later. Three were fed 5 pounds of linseed meal and slaughtered one hour later. Four were fed 5 pounds of linseed meal each and slaughtered three hours later, and five were fed 5 pounds of linseed meal each and slaughtered eight hours later.

The animal with a rumen fistula was used after the method of Schalk and Amadon<sup>10</sup>. In five trials she was fed 5 pounds of linseed meal one hour before the rumen was emptied through the rumen fistula, in five trials she was fed three hours before it was emptied, and in five trials she was fed eight hours before it was emptied. The rumen contents were replaced after they had been sorted over for boli, and the procedure was not repeated for at least 72 hours. A plug with a leather flange was kept in the opening of the fistula. This animal was in good condition after the experiments were finished, as shown in Figure 1.

In order to obtain information as to the course of the ground foods in the rumen and reticulum, three animals were given foods dyed with Sudan III and slaughtered as soon as possible after they had finished eating. Animal M236 was fed 5 pounds of a dyed grain mixture containing 3 parts ground corn, 1 part oats, and 2 parts linseed meal. This animal was slaughtered 15 minutes after she was given the feed. Animal M234 was fed 5 pounds of the same grain mixture that was fed M236, and slaughtered 20 minutes later. Animal 47 was fed 5 pounds of dyed ground corn and slaughtered 25 minutes later. The

<sup>10</sup> SCHALK, A. F., and AMADON, R. S. *Op cit*

Schalk and Amadon,<sup>5</sup> using the same method as Colin, reached a similar conclusion

Nevens<sup>6</sup> fed ground corn, dyed with Congo red 4 B, to cows which were killed shortly after feeding. In every instance practically all the corn was found in the rumen and reticulum mixed with the other contents. Very little of the ground corn was localized, and in only one case was it found farther than the rumen and reticulum. He states, "The mixing had been done almost as thoroughly as could be done by hand or by means of a mechanical mixer." The findings of Nevens were therefore somewhat contrary to those of Colin<sup>7</sup>, Schalk and Amadon,<sup>8</sup> and to the belief of Armsby<sup>9</sup> in that Nevens found practically all of the ground material in the rumen and reticulum mixed with the other contents.

Before the investigation reported in this paper was begun, the results of an experiment carried on by the dairy section of the Michigan experiment station had shown that dairy cows receiving 14 pounds of linseed meal per day for several months, without the addition of bulky material, remained healthy and showed no tendency to go off feed. The quantity of linseed meal and corn consumed are given in Table 1. The results of this experiment suggested that it may not be necessary to consider bulk in making up a grain mixture for dairy cattle.

TABLE 1 —Results of heavy consumption (pounds) of linseed meal and ground corn by cows during a long feeding experiment

Animal No	Lactation period	Average daily consumption from two to six months, inclusive, after calving <sup>a</sup>			Average daily consumption from calving to calving			Appetite
		Linseed meal	Corn	Total	Linseed meal	Corn	Total	
G-2	First.....	8.7	1.0	9.7	7.1	0.6	7.7	Good
	Second.....	10.6	5.1	15.7	8.7	4.3	13.0	Do
	Third.....	11.8	7.7	19.5	-----	-----	-----	Do
G-4	First.....	11.9	4.0	15.9	8.9	2.7	11.6	Do
	Second.....	14.3	7.3	21.6	10.5	4.8	15.3	Do
	Third.....	13.9	10.5	24.4	11.1	9.4	20.5	Off feed July 2-12, 1931. <sup>b</sup>
G-6	First.....	9.3	2.3	11.6	7.9	1.2	9.1	Good
	Second.....	11.1	5.1	16.2	8.0	3.2	11.2	Do.
	Third.....	11.7	8.2	19.9	9.0	6.8	15.8	Do
G-8	First.....	9.7	2.8	12.5	8.7	2.0	10.7	Do
G-10	First.....	9.0	2.6	11.6	5.7	1.4	7.1	Do
	Second.....	12.2	6.4	18.6	9.9	5.0	14.9	Do
	Third.....	11.5	8.3	19.8	-----	-----	-----	Do
G-12	First.....	10.2	4.1	14.3	8.2	2.8	11.0	Do
	Second.....	8.5	5.4	14.0	-----	-----	-----	Off feed for about 50 days.
G-14	First.....	11.0	3.5	14.6	9.4	3.0	12.4	Good
G-16	First.....	10.6	6.0	16.6	-----	-----	-----	Off feed July 2-4, 1931

<sup>a</sup> The average daily linseed-meal and ground-corn consumption from two to six months, inclusive, was taken because these animals were not placed on full feed for several weeks after calving, consequently food consumption for the first month was not as high as for the succeeding five months.

<sup>b</sup> Calved, June 29, 1931.

<sup>5</sup> SCHALK, A. F., and AMADON, R. S. PHYSIOLOGY OF THE RUMINANT STOMACH (BOVINE) STUDY OF THE DYNAMIC FACTORS. N. Dak. Agr. Expt. Sta. Bul. 216 48-49 1928.

<sup>6</sup> NEVENS, W. B. EFFECTS OF FASTING AND THE METHOD OF PREPARATION OF FEED UPON THE DIGESTIVE PROCESS IN DAIRY CATTLE. Jour. Agr. Research 36 785-788 1928.

<sup>7</sup> COLIN, G. Op. cit.

<sup>8</sup> SCHALK, A. F., and AMADON, R. S. Op. cit.

<sup>9</sup> ARMSBY, H. P. Op. cit.

TABLE 2 — *Boli recovered from three animals slaughtered one hour after being fed 2½ pounds of linseed meal and 2½ pounds of ground oats, and from three others slaughtered one hour after being fed 2½ pounds of linseed meal and 2½ pounds of ground corn*

ANIMALS FED LINSEED MEAL AND GROUND OATS		
Animal No <sup>a</sup>	Quantity of feeding recovered as boli	
	Grams	Per cent
204.....	3	0 13
C35.....	3	.13
171.....	6	26
ANIMALS FED LINSEED MEAL AND GROUND CORN		
262.....	10	0 44
128.....	198 5	8 75
191.....	56 7	2 50

<sup>a</sup> All animals trucked to place of slaughterTABLE 3 — *Boli recovered from animals slaughtered 1, 3, and 8 hours after being fed 5 pounds of linseed meal*

ANIMALS SLAUGHTERED ONE HOUR AFTER FEEDING		
Animal No	Quantity of feed recovered as boli	
	Grams	Per cent
26.....	113 4	5 00
223 <sup>a</sup> .....	453 6	20 00
256.....	510 3	22 50
ANIMALS SLAUGHTERED THREE HOURS AFTER FEEDING		
253.....	113 4	5 00
217 <sup>a</sup> .....	313 0	13 80
189 <sup>a</sup> .....	83 0	3 66
233 <sup>a</sup> .....	300 0	13 23
ANIMALS SLAUGHTERED EIGHT HOURS AFTER FEEDING		
231 <sup>a</sup> .....	1 0	0.04
M250 <sup>a</sup> .....	28 4	1 25
153 <sup>a</sup> .....	17 0	75
184 <sup>a</sup> .....	12 0	53
M266 <sup>a</sup> .....	5.0	.22

<sup>a</sup> Trucked to place of slaughter.

When 5 pounds of linseed meal was fed and the animals were slaughtered 1 hour, 3 hours, and 8 hours after feeding (Table 3), it was found that the rumen had considerable ability to break down and dissolve the boli (Figs 2 and 3) When 5 pounds of linseed meal was fed and the animals were slaughtered one hour later the largest amount recovered was 22 5 per cent, but when the animals were slaughtered eight hours after feeding the largest amount re-

feed of the five animals that received 5 pounds of ground oats and the five that received 5 pounds of ground corn and were slaughtered one hour after feeding, was dyed with Sudan III

### RESULTS

No boli were recovered from the rumen of the five animals that were fed 5 pounds of ground oats and slaughtered one hour later. The dyed oats were found fairly well distributed throughout the contents of the rumen. Likewise, no boli were found in the five animals that received 5 pounds of ground corn and were slaughtered one hour later; yet corn is generally considered a heavy feed

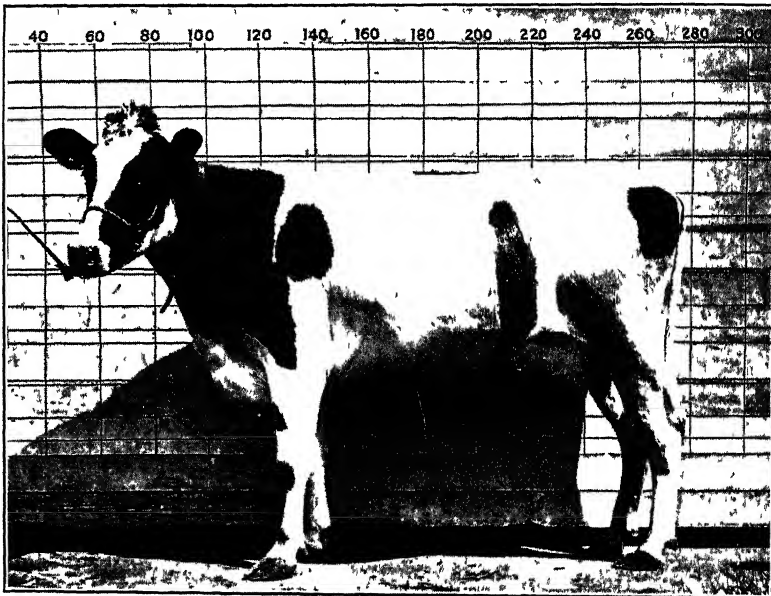


FIGURE 1—Cow No 238 with rumen fistula after the 15 trials reported in this investigation were finished. The plug has been removed in order to show the opening of the fistula

The weights of the boli recovered from the various other ground feeds are shown in Tables 2 and 3

When 2½ pounds of linseed meal was fed with 2½ pounds of ground oats and ground corn, respectively, the ground oats had the effect of separating the feed material, as is shown by the fact that much less of the mixture of ground oats and linseed meal was recovered than of ground corn and linseed meal (Table 2). However, a mixture of equal parts of ground corn and linseed meal would be about the heaviest and stickiest mixture that a farmer would ever use. In the three trials with this mixture only from 0.44 to 8.75 per cent of the concentrates was recovered in the form of boli when the animals were slaughtered one hour after feeding.

average percentage of linseed meal recovered in the eight tests was 16.4. In the 9 tests in which the boli were recovered three hours later it was 8.2 per cent, and in the 10 tests in which boli were recovered eight hours later it was 0.6 per cent.

In the three trials in which the animals were slaughtered 15, 20, and 25 minutes after the dyed feed was offered, no feed was found in the omasum or abomasum. In the case of M234 and 47, which were slaughtered 20 and 25 minutes after being fed, the feed was fairly well mixed with the rumen contents.

TABLE 4 — *Boli recovered from animal No. 238, with rumen fistula, in 15 trials after feeding 5 pounds of linseed meal and having the rumen emptied 1, 3, and 8 hours after feeding*

Trial No	1 hour after feeding		Trial No	3 hours after feeding		Trial No	8 hours after feeding	
	Grams	Per cent		Grams	Per cent		Grams	Per cent
1.....	611 0	26.94	6.....	21 0	0.93	11.....	17 0	0.75
2.....	323 0	14.24	7.....	160 0	7.05	12.....	9 0	.40
3.....	125 0	5.51	8.....	312 0	13.75	13.....	6 0	.26
4.....	562 0	21.78	9.....	121 5	5.36	14.....	18 0	.79
5.....	278 0	12.36	10.....	252 0	11.11	15.....	21 2	.91

In the tests in which five animals received 5 pounds of ground dyed oats and five received 5 pounds of ground dyed corn one hour before slaughter, there were two instances in which the dyed food was found in all four compartments. In one case the ground oats were found as far as the abomasum and in three cases as far as the omasum. However, the amounts found in both omasum and abomasum were insignificant.

#### DISCUSSION OF RESULTS

In analyzing the results obtained in this investigation it should be kept in mind that it is more than likely that the strength of contraction of the walls of the rumen varies somewhat in different animals, so that the material fed would be acted on more severely in some than in others. Moreover, the strength of contraction in the same animal may vary from time to time, as is shown by the varying results obtained from the animal with the rumen fistula. The hunger of the animals at the time of feeding would also influence the rate at which they would consume their food, and this in turn would affect the number and size of boli formed by deglutition. It should also be noted that there was as much variation in the weight of boli recovered from the animal with the rumen fistula as there was from the slaughtered animals. This would seem to eliminate any factor introduced by trucking the animals to the place of slaughter. In these studies no boli were noticed in either the omasum or the abomasum.

From the results of this investigation it would seem that the rumen has the ability to break up and dissolve boli even of the most cohesive feeds. The animals used in the tests mixed the feeds fed them with the roughage material of the rumen. Bulk therefore appears to be unnecessary in the grain mixture except possibly in the case of high-producing test cows that are being very heavily fed.

The results of this investigation indicate that the ground feeds all go to the rumen and reticulum and are mixed with the contents of these compartments. Once mixed with the rumen contents, they

covered was 1 25 per cent Very similar results were obtained with the animal having the rumen fistula (Table 4)

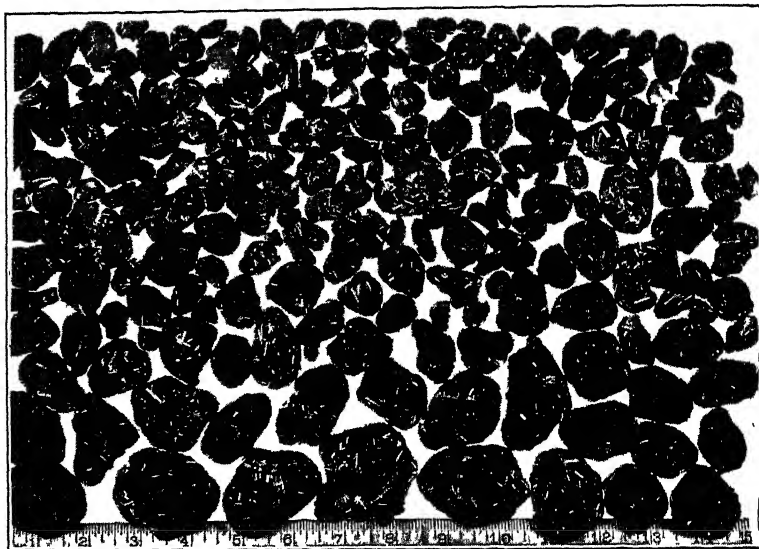


FIGURE 2—Bolus recovered from animal 256, fed 5 pounds of linseed meal and slaughtered one hour after feeding



FIGURE 3—The approximate decrease of the bolus of linseed meal in three (B) and eight (C) hours after feeding as compared to one hour (A) after feeding

By combining the results of the experiments in which linseed meal was fed and the animals slaughtered one hour later (Table 3) with the results of the experiments in which linseed meal was fed and the rumen emptied one hour later (Table 4), it will be found that the

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## CYTOLOGIC AND GENETIC STUDIES OF VARIABILITY OF STRAINS OF WHEAT DERIVED FROM INTERSPECIFIC CROSSES<sup>1</sup>

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### INTRODUCTION

The possibilities of combining the desirable characters of the *vulgare* and emmer groups of wheat in a single variety have received considerable attention from plant breeders. Special emphasis has been given to the breeding of a wheat that would combine the resistance to black stem rust (*Puccinia graminis tritici* Erikss. and Henn.) of the emmer group with the milling and baking qualities and other desirable characters of the *vulgare* group. At the Minnesota Agricultural Experiment Station a cross of Marquis with a highly rust-resistant variety of durum called Lumillo was made in 1914, from which Marquillo was produced. This variety possessed 42 chromosomes, high-yielding ability, resistance to stem rust, and stiff straw. When subjected to milling and baking tests, Marquillo wheat appeared to yield a flour which could be converted into bread that was satisfactory in every particular except perhaps in the matter of color.

Marquillo was a distinct step in advance, but there was still room for improvement. Accordingly, homozygous rust-resistant lines from the cross of Marquis  $\times$  Lumillo were crossed with Kanred  $\times$  Marquis selections, which excelled in agronomic characters. Minnesota 2303 is a selection from this cross which gives considerable promise, being rust resistant, possessing desirable agronomic characters, and apparently milling and baking qualities equal to those of Marquis.

The investigators at the University of Minnesota realized that Marquillo exhibited somewhat greater variability in agronomic characters than such varieties as Marquis. Cytological research was started in an endeavor to determine the germinal stability of this variety. As the work progressed it seemed desirable to add Marquis to the studies for purposes of comparison and Minnesota 2303 was included in a part of the study because it has considerable promise of becoming a highly desirable economic variety. The results are reported in this paper.

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<sup>2</sup> The writer wishes to express his sincere appreciation to Dr H K Hayes for kindly suggestions and criticisms during the course of the experiment and preparation of the manuscript. Also, acknowledgments and thanks are due Dr F J Stevenson for aid and valuable suggestions during the earlier part of the investigations. Thanks are due Dr Hannah C Aase for helpful suggestions and criticism of the manuscript.

await their turn at being exposed and washed to the omasum and abomasum. These observations concerning the course of ground feeds are in harmony with those obtained by Nevens<sup>11</sup> but not with those of Colin<sup>12</sup>, and Schalk and Amadon<sup>13</sup>. The results of Colin and of Schalk and Amadon were obtained with animals having rumen fistulas, whereas those of Nevens and those of the present writers were obtained with slaughtered animals. Studies upon the course of feed similar to those of Colin, and of Schalk and Amadon were not made, since by their method it is necessary to remove a portion of the rumen contents, which might produce abnormal effects on the course of feeds.

Feeders often note that when heavy rations are fed, the animals show a greater tendency to go off feed than when light rations are fed. However, the results of the experiments herein reported seem to show that the reason for the cows going off feed may not be a lack of bulk in the grain mixture.

In order to obtain bulk in light grain mixtures farmers frequently purchase bran, although it is generally possible to obtain the quantity of protein found in bran in cheaper form in other high-protein concentrates. Such feeds as beet pulp, ground oat hulls, and ground alfalfa are sometimes added to ready-mixed feeds for the purpose of supplying bulk, and these cheap materials are thereby sold at grain prices.

In the light of the experiments herein reported it does not appear to be good practice for farmers who are feeding for economical milk production to purchase bran or any other feed to add bulk to the grain mixture when the protein of such feeds is more expensive than that in other protein concentrates. The placing of oat hulls, beet pulp, and ground alfalfa in commercial feeds for the purpose of adding bulk can not be justified. These feeds are primarily roughages and when they are placed in the grain ration they compete with the roughages produced on the farm.

#### SUMMARY

All the grains consumed appeared to pass directly to the rumen and reticulum, where they were mixed with the contents of the rumen.

When 5 pounds of linseed meal was fed and the animals were slaughtered one hour afterwards, from 5.0 to 22.5 per cent of the feed was recovered in the form of boli. However, when 5 pounds of linseed meal was fed and the animals were slaughtered eight hours afterwards only from 0.04 to 1.25 per cent of the feed was recovered in the form of boli.

The results obtained on an animal with a rumen fistula checked very closely with those obtained on the slaughtered animals.

Since boli are broken up and mixed with the roughage in the rumen and reticulum, it appears unnecessary to consider bulk in the make-up of a grain mixture for dairy cattle fed for economic milk production.

<sup>11</sup> NEVENS, W. B. Op cit.

<sup>12</sup> COLIN, G. Op cit.

<sup>13</sup> SCHALK, A. F., and AMADON, R. S. Op cit.

Huskins (9) found that in speltoids of type A furnished him by Akerman the gametes all had 21 chromosomes. No deviation from the normal arrangement of 21 bivalents was found in the normal plants. The heterozygous speltoid plant showed a trivalent and a univalent in many of its pollen mother cells, though naturally it was not always possible to prove the existence of both in the same cell. The homozygous speltoid plant showed a quadrivalent in many of its pollen mother cells and a trivalent was seen in two of them.

Huskins (9) examined speltoids of the B type furnished by Nilsson-Ehle. The normal plants have the normal chromosome number and normal divisions. Each of the heterozygotes has only 41 chromosomes. In almost every case these formed 20 bivalents and 1 univalent. According to Huskins, Akerman has described a strain that differs from the ordinary B type strains in having produced some moderately vigorous and fertile homozygous speltoid progeny instead of only sterile dwarf ones. Huskins (9), from 30 seeds of a descendant homozygous speltoid plant sown in November, 1926, obtained 10 homozygous speltoid progeny. One of these was examined cytologically and found to have only 41 chromosomes. The behavior of this one was different, however, from that found in the 41-chromosome heterozygote of preceding strains. The odd chromosome was seen dividing on the plate during the anaphase in only about 75 per cent of the cells examined. A trivalent instead of a univalent was seen in a very large number of cells. These trivalents were of various shapes. In no case was a trivalent found to be accompanied by one univalent in this plant. Split univalents going at random to either pole were seen in many second divisions.

Huskins (9) found the heterozygous speltoid plants of type C to have 43 chromosomes, the homozygous speltoids were all rather weak, more or less sterile, and had 44 chromosomes. At the first metaphase of the heterozygous speltoids the chromosomes were believed to be arranged usually as 20 pairs and 1 trivalent. The method of pairing of the trivalent was usually end to end. All the 44 chromosome C types were very irregular cytologically.

Huskins (9), in studying the speltoids, noted further abnormalities. In one case the loss of approximately half a chromosome was noted and in another two of the members of a trivalent showed distinct subterminal constrictions. One of Huskins' illustrations of the metaphase of the first division shows a pair off the equatorial plate.

Huskins (8) reports the occasional occurrence of laggard and vagabond chromosomes in varieties of *Avena sativa*. He also reports that all the type 1 fatuoids had 42 chromosomes. In the normal segregates cytological conditions were found to be more irregular than in normal pure-line varieties of *A. sativa*. The presence of laggard and vagabond chromosomes was noted more frequently, and fewer cells showed perfectly regular splitting of the bivalents in the first anaphase.

#### MATERIALS AND METHODS

In 1929 seed of Marquillo was space planted in 5-foot rows so as to make possible individual plant studies. Two spikes on each of the resulting plants were bagged and both bagged and nonbagged spikes were harvested the following fall. Later the percentage of fruitfulness was determined on both the bagged and nonbagged heads. Cytologic

## REVIEW OF LITERATURE

The literature on the cytology of *Triticum*, especially as it pertains to phylogeny, has been reviewed by Aase (1)<sup>3</sup>. Watkins (18) gives a critical account of the present knowledge of the origin and genetic relationship of the wheat species, and their cytological and genetic behavior when crossed. For a more extensive review of literature than is given here the reader is referred to their articles. In this paper only that part of the literature is considered which has a direct bearing on the problem under discussion.

Huskins (10) states that his colleague, J. Philp, has found chromosome irregularities in the  $F_1$  plants resulting from crosses of hexaploid oats. Huskins (10) also found irregular chromosome behavior in a cross between *Triticum vulgare* and *T. spelta* made by Nilsson-Leissner. Huskins states that Nilsson-Leissner found this cross to segregate commonly for type of glume in a 3:1 ratio, but sometimes in almost the reverse proportions, and a number of abnormal types appeared.

Sapehin (15), working with pure lines of wheat and  $F_1$  crosses between 42-chromosome wheats, distinguished two types of anomalies which occur during meiosis. The first of these types is characterized by the occurrence of univalents, the second type by a disorderly arrangement of the chromosomes which readily vacuolize. Sapehin states that by cytological investigation he has proved that spore formation does not proceed normally in all pure lines, but that there occur anomalies of the first type in numbers from a fraction of 1 per cent of the cells to several per cent. He makes the following statement (15, p. 164).

Of special interest in this regard is the best yielder among the author's pure lines of soft spring wheat, *T. militurum* 00180, showing every year up to 20-30%, and sometimes even up to 50-60%, of spore mother cells of anomalies of the second type \* \* \* as the author's investigations have shown, spore formation in  $F_1$  00180  $\times$  other 42-chromosome wheats displays on the whole normal pictures, no matter whether 00180 has been the mother or the father plant.

Sapehin found that many anomalies were produced in crosses of Ukrainian lines with "related" or "identical" varieties from Afghanistan and eastern Siberia.

On the basis of ratios in which the heterozygous speltoids segregate, Nilsson-Ehle (13) divided speltoids into three types, A, B, and C. In type A the ratio approximates 1:2.1 (564 normal segregates:757 heterozygous speltoids:165 homozygous speltoids), but the homozygous speltoids were deficient. He found that heterozygotes of type B gave very few homozygous speltoids, and the heterozygotes were more numerous than would be expected on a 1:2:1 basis. A total ratio of 317 normal segregates:1,300 heterozygous speltoids:13 homozygous speltoids was obtained. His heterozygotes of type C again gave very few homozygous speltoids but gave more normals than heterozygotes, a total of 491 normal segregates:456 heterozygous speltoids:8 homozygous speltoids. However, this difference between the normals and heterozygotes is not great. Nilsson-Ehle found that in most cases the three types A, B, and C are easily distinguished by their different ratios, but occasionally a ratio may leave the matter doubtful, for example, 120:188:1.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 831.

were made, was obtained from plants spaced 3 inches apart in 5-foot rows

The progeny of 23 of the 30 plants studied cytologically were grown in 1930 for the purpose of correlating morphological and size variations under field conditions with previously determined cytologic irregularities and also for the purpose of determining the amount of natural crossing. Only seed from spikes that had been bagged the previous year was used in planting. The plantings were made in 5-foot rows 4 inches apart and the seed was spaced 4 inches within the row. First, two rows of Ceres were sown, then two days later a row of Marquillo, and two more rows of Ceres. This method of planting provided for two rows of Ceres on one side of Marquillo planted two days earlier and two rows on the other side planted on the same date as Marquillo. During the summer two heads of each plant of Marquillo were covered before pollination had occurred and the remaining spikes were left uncovered. Notes were taken on emergence, survival, number of spikes per plant, height of individual plants, fruitfulness, weight of seed per plant, and certain qualitative characters.

Only the outside florets of the noncovered seed were used in determining percentage of fruitfulness. Coefficients of variability were calculated for the percentage of fruitfulness in outside florets of noncovered spikes, center florets of noncovered spikes, outside florets of covered spikes, and center florets of covered spikes. The coefficients of variability were  $16.5 \pm 1.30$ ,  $117.2 \pm 6.16$ ,  $22.3 \pm 1.78$ , and  $131.9 \pm 12.96$ , respectively. These results show that the center florets in noncovered and covered spikes varied greatly in percentage of fruitfulness and that the outside florets of the bagged spikes varied more than nonbagged spikes. The average percentage of fruitfulness in the outer florets of the noncovered spikes was  $85.1 \pm 0.92$  as compared to  $18.9 \pm 2.09$  for center florets,  $78.8 \pm 1.33$  for outer florets of covered spikes, and  $12.9 \pm 2.10$  for center florets of covered spikes. Because of these results only the outside florets of noncovered spikes were used as a measure of fruitfulness.

In tests conducted in the greenhouse during the winter of 1929-30, progeny of eight plants of Marquillo in the seedling stage showed pronounced resistance to *Puccinia graminis tritici*, physiologic form 21. Ceres tested at the same time showed susceptibility. To determine the amount of natural crossing in these lines, seeds from bagged spikes of these plants were planted in the manner outlined above. During the summer two heads of each plant were bagged and those remaining were left uncovered. The following winter progeny from both covered and noncovered spikes were tested in the greenhouse for resistance to black stem rust, form 21. Since susceptibility is dominant in a cross between Marquillo and Ceres the plants resulting from cross fertilization were readily distinguishable.

#### TERMINOLOGY

Certain terms used in discussing the different chromosomal aberrations found occurring during microsporogenesis may need some explanation. Nonorientation of bivalents has been applied to the occurrence of a bivalent or bivalents off the equatorial plane just prior to disjunction of the main group of bivalents during metaphase.

material was taken from plants selected at random and the entire florets of spikelets were killed and fixed in Allen's modification of Boun's solution. All cytologic studies were made from permanent paraffin sections stained by using Newton's iodine-gentian-violet method as described by Huskins (8).

Sections were cut  $15\mu$  thick and lengthwise of the spikelet, with the exception of a very little of the earlier prepared material, which was cut transversely. This method of handling the material provided a side view of a majority of the achromatic figures of the microsporocyte, greatly facilitated the studies, and enhanced the ease with which they could be made, as a greater number of microsporocytes or microspores could be studied in one section. Sufficient end views of achromatic figures were obtained for purposes of comparison and the making of chromosome counts. The use of entire spikelets was also particularly advantageous as sections were frequently found which showed a number of the stages of sporogenesis. For example, a single section has been found showing the prophase of the first division, diakinesis, metaphase of first division, diads, and nearly mature microspores.

All counts of chromosomes were made from a side or end view of the metaphase or early anaphase of the first meiotic division of microsporogenesis. The number of chromosomes was determined by making counts from those cells having the chromosomes distinctly separated, and, therefore, easily distinguishable (Pl. 4, A, D, and F). In most plants it was possible to get good counts from both side and end views of the metaphase as well as the anaphase. Determination of chromosome number from side views was made by the aid of camera-lucida drawings (Pl. 4, C and E).

The studies of the occurrence of micronuclei were made on immature microspores still grouped together in tetrads in practically all cases, but in a few of the plants the microspores had broken apart and no longer exhibited this arrangement. However, in these latter plants the nucleus stained well and the microspores did not present the wrinkled condition characteristic of mature pollen grains. Microspores through which the knife had passed were not included in the counts, and only micronuclei well embedded in the cytoplasm were counted.

Of the material grown in 1929, 30 plants were studied both cytologically and genetically. The progeny of three of these plants were selected for further cytologic and genetic studies the next year, for the following reasons. Plant 407-12-3 had only 41 chromosomes, plant 407-17-13 had the normal number of chromosomes but showed considerable irregularity in chromosome behavior during microsporogenesis, and plant 407-12-24 possessed 42 chromosomes and was found to have considerable regularity of chromosome behavior during microsporogenesis. Marquis was included in these studies for purposes of comparison. All seeds were sprouted in germinators, transplanted into pots in the greenhouse, and later the resulting plants were transplanted into the field, being placed in 5-foot rows, 1 foot apart and the plants spaced 6 inches within the row. Correlated data were kept on cytologic phenomena, germination, vigor of plants, survival, number of spikes per plant, height of individual plants, fruitfulness, weight of seed per plant, and certain qualitative characters. The material from which cytologic studies on Minnesota 2303

nificance in relation to the fragmentation reported later, as it is extremely doubtful whether these parts of chromosomes would be passed on to the next generation. The behavior of the lagging chromosomes during the metaphase of the first division, anaphase of the second division, and telophase of the second division strongly indicates that these small clumps of chromatin are the result of lagging chromosomes which have formed micronuclei, and they will be considered as such in the remaining part of this paper. Complementary micronuclei are of common occurrence in young microspores of interspecific wheat hybrids which are semihaploid. (Kihara (11), Watkins (17), Aase (1) )

Of 185 immature microspores examined, 30.3 per cent showed micronuclei, which, as will be shown later, is strong evidence that the microsporocytes had one univalent chromosome during sporogenesis. Very infrequently cases were found in which a cell wall had been laid down between the macronucleus and the micronucleus, thus forming miniature microspores.

Plant 407-12-3 was the other one of the 32 examined that had 41 somatic chromosomes, as determined by counts made from both the anaphase of the first division and side view of the metaphase. Plate 4, C, shows drawings of 20 bivalents and 1 single made from the side view of the metaphase of the first division. The counts on chromosome number are further substantiated by the fact that of the 63 pollen mother cells studied in the metaphase stage 57, or 90.5 per cent, showed the single chromosome. Moreover, it was possible to find lagging chromosomes in the late telophase of the second division that gave indications of forming micronuclei. Of the 667 immature microspores observed in the tetrad stage, 172 possessed both a macronucleus and a micronucleus. This is 25.8 per cent of the number examined. Of this 25.8 per cent, the majority had only 1 micronucleus, but infrequently 2, 3, or 4 were present, the latter being the highest number found in 1 microspore. More than 1 micronucleus in a single microspore is probably due to some of the chromosomal aberrations discussed later.

The occurrence of plants within a variety having one less than the normal chromosome number raises the question as to their origin. It will be remembered that Marquillo is descended from a cross between Marquis (42 somatic chromosomes) and Iumillo (28 somatic chromosomes). Are the 41-chromosome plants occurring in the fifteenth generation after the cross descendants of plants which have not attained the chromosome number normal to *Triticum vulgare* or are they descendants of 42-chromosome plants and owe their origin to irregular behavior of chromosomes during meiosis? The chromosomal aberrations which are described below may be of some aid in answering this question.

#### OCCURRENCE OF MICRONUCLEI IN 42-CHROMOSOME PLANTS

Thirty plants of Marquillo and Minnesota 2303 and 27 plants of Marquis were studied for the occurrence of micronuclei. The data given in Table 1 show that  $2.8 \pm 0.16$  of the microspores of Marquillo showed this condition, and  $0.8 \pm 0.04$  of those of Minnesota 2303, and  $0.8 \pm 0.06$  of those of Marquis. One plant of Marquillo had as high as 7.6 per cent of the microspores showing micronuclei and another as low as 1.1 per cent, whereas the range in Minnesota 2303 was from 0.3

of sporogenesis (Pl 1, C and D) Nonconjunction is used to signify the occurrence of a univalent or univalent chromosomes during the metaphase of the reductional division, when presumably the homologous mate or mates of this univalent or these univalent chromosomes are present (Pl 3, D) Polyvalence has been employed to designate the union of three or more chromosomes during the metaphase of the reductional division (Pls 2, A, and 3, F) Predisjunction is used to denote the disjunction of a bivalent or bivalents in advance of the main group of conjugated chromosomes during metaphase of the reductional division (Pl 3, E)

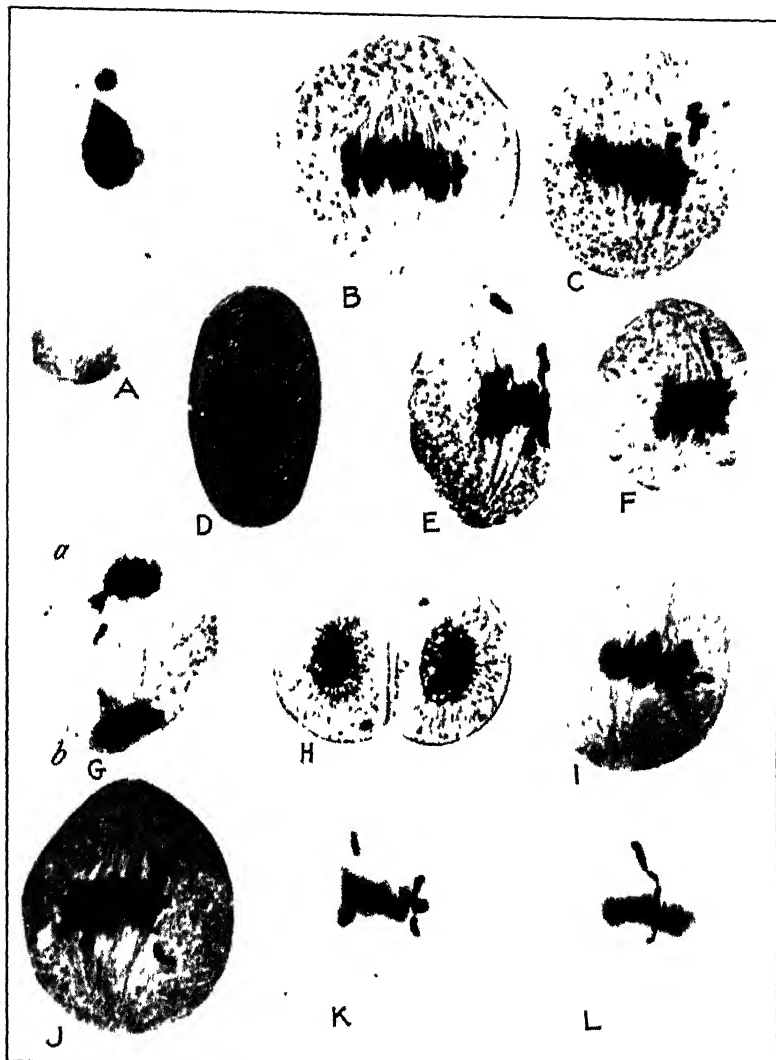
## CYTOLOGIC STUDIES OF ABERRATIONS

### CHROMOSOME NUMBERS AND THE BEHAVIOR OF UNIVALENTS IN 41-CHROMOSOME PLANTS

The number of chromosomes was determined for 32 plants of Marquillo grown in 1929, and 27 plants of Marquis grown in 1930. The plants of both Marquillo and Marquis were unselected, and may be considered as representative in so far as samples composed of such small numbers can be representative. All the plants of Marquis and all but 2 of the 32 plants of Marquillo were found to have the normal somatic chromosome number of 42. The somatic chromosome number of the other 2 plants of Marquillo, culture numbers 407-15-17 and 407-12-3, was 41.

A study of the different stages of microsporogenesis of these 41-chromosome plants is of interest not only because it furnishes further evidence as to the authenticity of the counts, but also because it supplies information as to the behavior of the univalent chromosome during sporogenesis. This information is valuable in an analysis and an interpretation of the significance of the chromosomal aberrations which are discussed later.

In plant 407-15-17 microsporocytes showing different stages were available—diakinesis, metaphase, late anaphase of the second division, telophase of the second division, and young microspores not having undergone complete separation (Pl 5, A-F). It was impossible to identify the univalent chromosome in the diakinesis stage, as can be seen from an examination of Plate 5, A. All but three of the chromosomes in this figure showed their paired nature, and any of these three may well have been the univalent. The univalent chromosome was clearly distinguishable in 84 of the 101 pollen mother cells examined during the metaphase of the first division. Plate 5, B, shows 20 pairs lined up on the equatorial plane and 1 single and a fragment not on the equatorial plane. Plate 4, F, is an end view of the metaphase of the same plant, showing 20 bivalents and 1 univalent chromosome. Diads of the first division and metaphases of the second division were not available, but the sections showed some microsporocytes in the anaphase of the second division, and lagging chromosomes were present (Pl 5, C). Probably only 2 of the 4 chromosomes not at the poles are due to the univalent chromosome of the metaphase. Plate 5, D-F, shows the behavior of the lagging chromosomes during reconstruction of the nuclei after the second division. In the left half of Plate 5, E, is shown a chromosome which has been divided transversely, presumably because of the formation of the cell plate. This is believed to be of very little sig-



Stages in the meiotic divisions, microsporocytes, photomicrographs ( $\times 1,500$ )

- A —Extrusion of karyotin, early leptotene stage, Marquillo
- B —Regular equatorial plane, metaphase of first division, Marquillo
- C —Nonorientation of a bivalent, metaphase of first division, Marquillo
- D —Nonorientation of a bivalent, metaphase of first division, Marquis
- E —Nonconjunction or predisjunction and nonorientation, metaphase of first division, Marquis
- F —Predisjunction and nonorientation, metaphase of first division, Marquis
- G —Lagging chromosomes *a*, reductional division only, *b*, reduction division and equational division, telophase of first division, Marquis
- H —Micronuclei, microspores arranged in tetrads, Marquillo
- I —Univalent on the equatorial plane, metaphase of first division, 41-chromosome plant of Marquillo
- I —Univalent off the equatorial plane, metaphase of first division, 41-chromosome plant of Marquillo
- K —Trivalents, metaphase of first division, 41-chromosome plant of Marquillo
- L —Trivalents, metaphase of first division, 41-chromosome plant of Marquillo

per cent to 1.7 per cent and that of Marquis from 0 to 2.2 per cent. There was only one plant of Marquis which failed to show the occurrence of micronuclei. As can be readily seen from an examination of the probable errors of the means, the difference noted between Marquis and Marquillo is statistically significant, and the means for Marquis and Minnesota 2303 are the same. In regard to the percentage occurrence of micronuclei, Marquis had a coefficient of variability of 53.7, Marquillo 46.0, and Minnesota 2303, 38.7. The data for Marquillo were based upon an examination of 18,644 microspores, those for Marquis upon 15,844 microspores, and those for Minnesota 2303 upon 20,944 microspores, making an average of somewhat over 500 microspores per plant. Only a few plants were included from which less than 500 microspores were studied, the lowest number was 382.

TABLE 1—Average percentage and coefficient of variability of microspores showing micronuclei

Variety or culture	Number of plants	Microspores		
		Total	Micronuclei	
			Average	Coefficient of variability
Marquillo.....	30	18,644	2.8±0.16	46.0
Marquis.....	27	15,844	8±0.06	53.7
Minnesota 2303.....	30	20,944	8±0.04	38.7

It should be noted that 30.3 per cent of the microspores showed micronuclei in plant 407-15-17 and that 25.8 per cent revealed this phenomenon in plant 407-12-3. Micronuclei were present in 2.8 per cent of the microspores of the other 30 plants of Marquillo and in 0.8 per cent of the microspores of Minnesota 2303 and Marquis. Plate 1, H, is a photomicrograph of microspores of Marquillo showing micronuclei. It has been shown that the micronuclei in the 41-chromosome plant probably are formed from the univalent chromosome. The origin of the micronuclei in the 42-chromosome plants remains to be determined.

#### EXTRUSION OF KARYOTIN

Plate 1, A, is a photomicrograph of a very young microsporocyte of Marquillo culture, 407-15-17 (41 somatic chromosomes), in which karyotin is being extruded from the nucleus. Both the nucleus and extruded karyotin were deeply embedded in the cytoplasm and, therefore, could not have been touched by the microtome knife. At first it was thought that the extruded karyotin might be the nucleus, but as can be seen from the photomicrograph, which also shows the nucleolus being extruded into the nuclear cavity, the two differ materially in structure and staining reaction. Of the cells studied for this phenomenon, some in later stages showed a nucleolus in the nuclear cavity, but no cells were found in which the nucleolus was in the surrounding cytoplasm. The amounts of the karyotin varied from those as large as that shown in Plate 1 to those approximately one-third of that size. Plant 407-15-17 showed extrusion of larger

amounts of karyotin (pl 1, A) in 12 of the 431 cells examined and of smaller amounts (pl 3, A) of the same material in 28. This abnormality occurred in 2.8 and 6.5 per cent, respectively, of the microsporocytes.

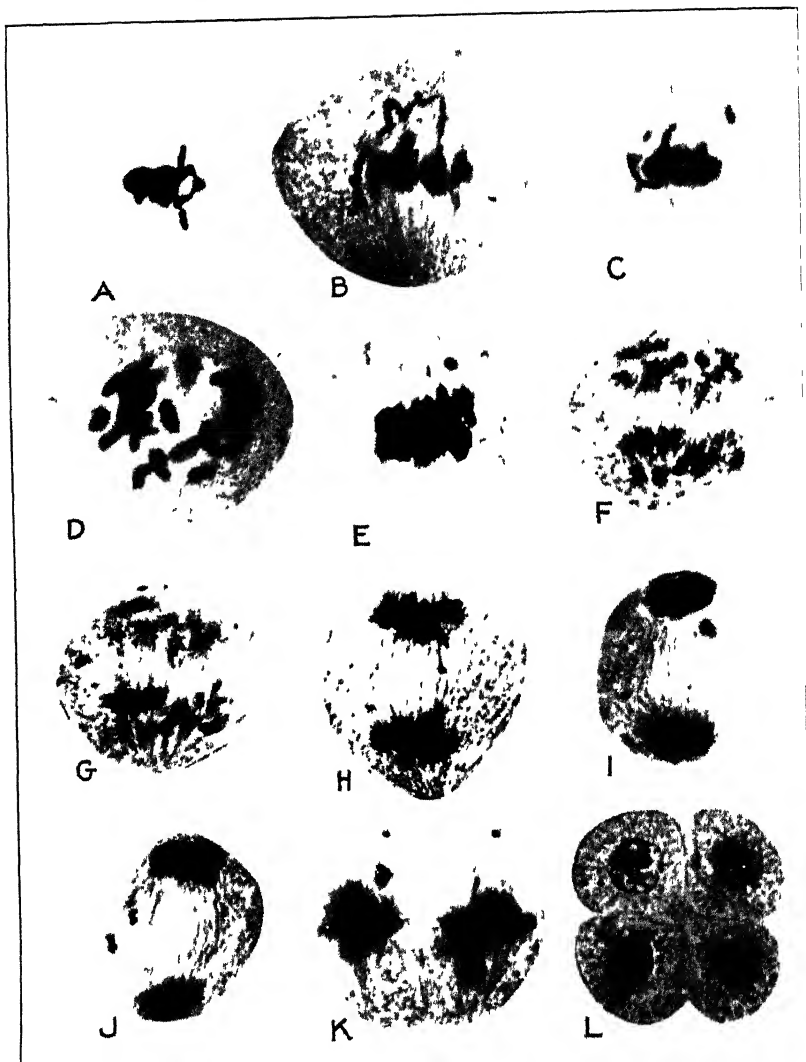
In order to determine whether this phenomenon was due to the abnormal chromosome number of plant 407-15-17 the same studies were made with plant 407-12-24 and a plant of Marquis. In plant 407-12-24, 0.5 per cent of the 432 microsporocytes examined exhibited large extrusions and 1.9 per cent smaller extrusions. The plant of Marquis revealed 2 out of 540 microsporocytes showing extrusion of smaller karyotin clumps and one of the larger size. Extrusion of karyotin has been reported in the sporocytes of *Crocus*, *Iris*, *Crepis*, *Oenothera*, and other plants, according to Sharp (16).

#### NONORIENTATION OF BIVALENTS

Between the diakinesis (pl 2, D, and 5, A) and metaphase (pl 1, B) stages there exists a transition period in which the nuclear cavity and nucleolus disappear and the chromosomes undergo transformation, losing the ragged and loose appearance shown in Plate 5, A, and becoming more definite in outline and compact, as shown in Plate 5, B. Examination of pollen mother cells showing this transition period does not indicate that the movement of the bivalents to the equatorial plane is a strictly methodical procedure. Some of the chromosomes may be at the equatorial plane while others are clearly defined in the surrounding cytoplasm. It seems, then, that at least in some cells all chromosomes do not undergo this transformation with the same degree of rapidity, nor do all seem to arrive on the equatorial plane at the same time. If such a supposition is true, it is to be expected that in some cases bivalent chromosomes should be found off the equatorial plane when all the other chromosomes are lined up and ready to undergo disjunction.

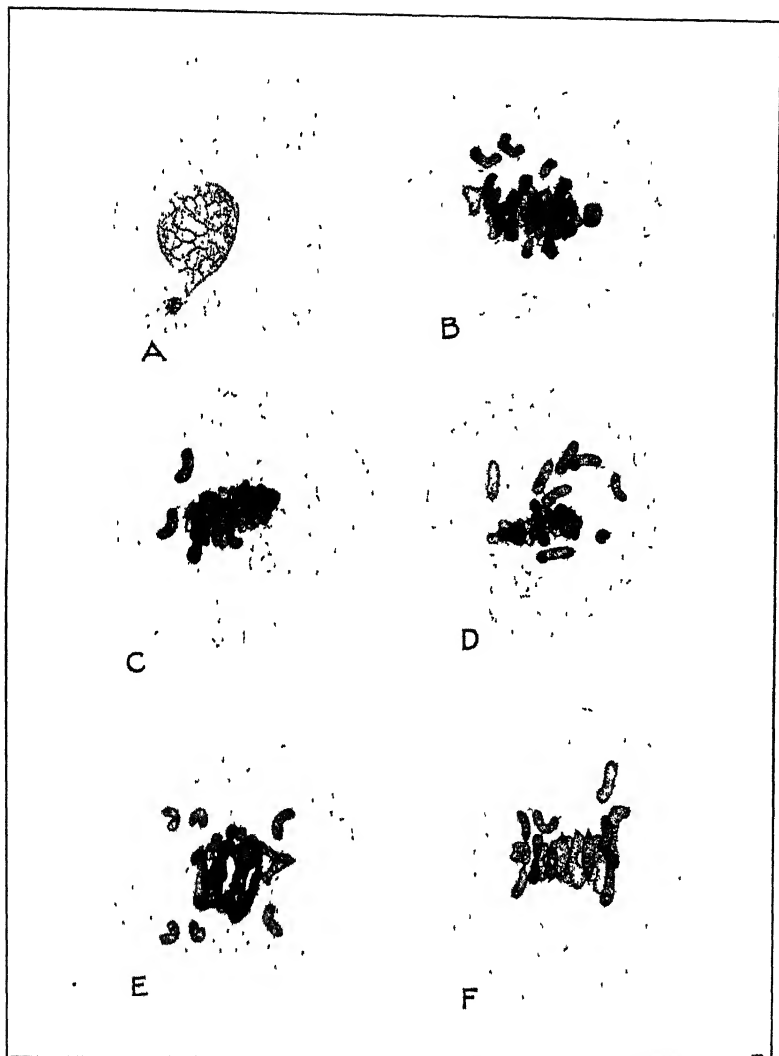
C and D of Plate 1 are photomicrographs of microspores of Marquillo culture 407-12-24 and Marquis culture 225-3 which exhibit this condition. As many as three bivalents in one cell have been found off the equatorial plane. This phenomenon has been termed nonorientation of bivalents. In these studies on nonorientation, only those cells showing bivalents not on the equatorial plane when the remaining chromosomes were definitely oriented and about to undergo disjunction have been considered as showing this phenomenon. Plate 1, B, is a side view of a Marquillo pollen mother cell showing all chromosomes lined up regularly on the equatorial plane. The frequency of the occurrence of nonorientation is of interest. An average of  $10.8 \pm 0.68$  per cent of the 2,830 Marquillo microsporocytes studied showed nonorientation of bivalents and an average of  $6.9 \pm 0.49$  per cent of the 3,327 Marquis microsporocytes revealed this aberration. The lowest percentage found in any plant of Marquillo was 3.8 and the highest was 28.6 per cent. The range in Marquis was from 1.8 to 21.4 per cent.

The question as to the subsequent behavior of nonoriented bivalents is important in connection with the point whether they result in the formation of micronuclei in the microspores. It seems possible that such bivalents might reach the equatorial plane late and undergo disjunction. If such were the behavior of these chromosomes, it would be expected that they could be found undergoing disjunction



Stages The meiotic divisions, microsporocytes, photomicrographs ( $\times 1,500$ )

- A — Polyvalence, probably hexavalents, metaphase of first division, 41 chromosome plant, Marquillo  
 B — Polyvalence off the equatorial plane, number of chromosomes involved is unknown, 41 chromosome plant, Marquillo  
 C — Trivalents showing end constriction, metaphase of first division, 42-chromosome plant, Marquillo  
 D — Fragment above nucleolus, diakinesis of first division, 41-chromosome plant, Marquillo  
 E — Fragment, metaphase of first division, 42-chromosome plant, Marquis  
 F — Fragmentation, anaphase of first division, 42-chromosome plant, Marquillo  
 G — Fragmentation, same cell as F at different level, anaphase of first division, 42-chromosome plant, Marquillo  
 H — Fragmentation, same plant as F and G, different cell, anaphase of first division, 42-chromosome plant, Marquillo  
 I — Fragment and single chromosome which has undergone only reductional division, telophase of first division, Marquillo  
 J — Fragment and single chromosome which has undergone reductional and equational division, same cell as I, invert for comparison, telophase of first division, Marquillo  
 K — Fragment in both diads, same plant as I and J, different cell, metaphase of second division, Marquillo  
 L — Fragment, upper left microspore, tetrad, 42-chromosome plant, Marquis



Stages in the meiotic divisions, microsporocytes, camera-lucida drawings ( $\times 2,000$ )

- A -- Extrusion of karyotin, early leptotene stage, 41-chromosome plant, Marquillo
- B -- Predisjunction and nonorientation, metaphase of first division, 42-chromosome plant, Marquillo
- C -- Predisjunction and nonorientation, metaphase of first division, 42-chromosome plant, Marquillo
- D -- Nonconjunction, eight univalents, metaphase of first division, 42-chromosome plant, Marquillo
- E -- Predisjunction, three bivalents, metaphase of first division, 42-chromosome plant, Marquillo
- F -- Trivalents, two univalents, metaphase of first division, 41-chromosome plant, Marquillo

during the anaphase of the first division in some of the microsporocytes. No well-differentiated cases of late disjunction of a bivalent were found. However, there were only a few microsporocytes in the proper stage for detecting this phenomenon, and as nonorientation occurred in only 10.8 per cent of the Marquillo microspores and in only 6.9 per cent of those of Marquis, bivalents undergoing late disjunction on the equatorial plane would not necessarily be found even though they were occurring. That a differentiation in the time of disjunction does occur is shown by Plate 3, E. At the right of the achromatic figure of this pollen mother cell are shown two bivalents. The chromosomes of one pair have pulled apart and are well started toward their respective poles, while the members of the other pair are still closely conjugated. It does not seem that the condition noted in Plate 3, E, would give rise to lagging chromosomes and hence micronuclei.

There also remains the possibility that these bivalents undergo disjunction even though they are not on the equatorial plane. B and C of Plate 3, are camera-lucida drawings offering evidence in support of this supposition. The two chromosomes in the upper left hand corner of Plate 3, B, are believed to have been loosely paired homologous chromosomes which have separated early. This conclusion is based on the fact that the two chromosomes closely resemble each other both in size and form and are in the same plane as regards their orientation on the achromatic figure. That this assumption is probably correct is further confirmed by the fact that the phenomena described are characteristics of early divided pairs whose members are on opposite sides of the main group of chromosomes as is shown in the same cell (pl. 3, B) and in a different microspore of the same plant (pl. 3, E). In contrast to these chromosomes are the nonconjugated chromosomes of Plate 3, D, which show no relationship to each other as regards their orientation in the achromatic figure. Plate 3, C, is also a camera-lucida drawing showing two chromosomes which it is believed are homologous mates that have disjoined off the equatorial plane. Since the disjoining of these two chromosomes, the lower has moved toward the lower pole and attained a position on the edge of the main group. In this particular cell it seems logical to suppose that the two chromosomes in question would reach their respective poles and be included in the reconstructed nuclei as they would have done had they lined up with the other bivalents. Other cells show this same phenomenon with the chromosomes in various positions. If this assumption that the members of bivalents can separate off the equatorial plane is correct, pairs should be found starting to show disjunction. Plate 1, F, which is a photomicrograph of a microsporocyte of Marquis, shows two chromosomes paired end to end which are apparently undergoing disjunction, being connected by only a small thread of chromatin. Two widely separated chromosomes in the same plane and on the same side of the equatorial plane are shown in Plate 1, E. This figure is also a photomicrograph of a microsporocyte of Marquis. The nonoriented bivalent of Plate 1, C, offers further evidence. This chromosome has undergone complete transformation in form, as can be seen from an examination of the photomicrograph. That its members are undergoing disjunction at the same time as the bivalents on the equatorial plane also seems probable as the free ends of the members are oriented toward opposite poles. The evidence supports the

supposition that the chromosome pairs at a certain period in transformation and development disjoin regardless of whether they are on the equatorial plane. It seems that the time at which this stage of development is reached may be correlated with the strength of association between the conjugants as exhibited by the type of pairing. For a discussion of the types of conjugation, which is not within the scope of this paper, see Aase (1).

Whether the nonoriented bivalents divide later than the oriented bivalents is of equal importance. It is doubtful whether the non-oriented bivalent shown in Plate 1, D, would disjoin as soon as the chromosome pairs on the equatorial plane, as it does not appear to have completely undergone the transformation that occurs between the diakinesis and metaphase stages of the first division. Although somewhat more regular in outline, it still is similar in form to some of the chromosomes in Plates 2, D, and 5, A. Then, this chromosome pair has the appearance of being a lagger in both transformation and orientation. If such is the true interpretation, this bivalent would not be expected to divide as soon as the chromosomes on the equatorial plane. Plate 1, G, may furnish some information on this problem. This photomicrograph of the telophase of the first division shows one univalent which has not undergone the second division and two separated chromosomes resulting from a univalent which has divided equationally. As shown here, chromosomes resulting from an equational division can be distinguished easily from these originating from a disjunctive division. Also, as is shown by the upper chromosome of Plate 1, G, the nonequationally divided chromosomes of this stage clearly show the split for the second division. It will be noted also that all three chromosomes are in the same plane, which strongly suggests that they represent separated homologous chromosomes which have first undergone a reductional division and then the lower resultant chromosome has undergone an equational division. The disjunction of the bivalent must have occurred later than that of those giving rise to the nuclei at the poles, otherwise the nondivided chromosome would be expected to have been included with the main group at the pole. Also, it seems logical to assume that the bivalent giving rise to the lagging chromosome was not oriented on the equatorial plane at the time disjunction occurred, because if it had been both resultant daughter chromosomes would probably have exhibited the same behavior. On the other hand, if disjunction had taken place nearer the pole to which the undivided single is in proximity, the other member as it traveled toward the opposite pole may have been expected to undergo the equational division that has actually occurred. That this is an equational division and not a reductional division may be readily determined by a comparison between it and the nonoriented pair in Plate 1, C. That the upper chromosome of the three in Plate 1, G, has not undergone the equational division may be determined by comparing it with the chromosomes at the poles in Plates 2, F, and 4, A, and with the equationally divided lagging chromosomes in Plate 5, C. Comparatively speaking, the condition described can be found frequently in both Marquis and Marquillo. Plate 2, I-J, are photomicrographs of the same microsporocyte of Marquillo taken at different levels showing about the same condition as Plate 1, G. To have the orientation of the two photomicrographs comparable either one or the other should be inverted. A chromosome which has not



Stages in the meiotic divisions, microsporocytes, camera-lucida drawings ( $\times 2,000$ )

- A —Fragmentation, 42 chromosomes, same cell as Plate 2, F and G, anaphase of first division, Marquillo  
 B —Fragmentation, 42 chromosomes, same cell as Plate 2, H, anaphase of first division, Marquillo  
 C —20 bivalents, 1 single, drawn from side view, metaphase of first division, Marquillo  
 D —21 chromosomes, end view, anaphase of first division, Marquillo  
 E —19 bivalents, 1 trivalent, drawn from side view, metaphase of first division, Marquillo  
 F —20 bivalents, 1 univalent showing end constriction, end view, metaphase of first division, Marquillo

The range of nonconjunction in Marquillo was from 0 to 13.0 per cent and in Marquis from 2.3 to 40.2 per cent. The number of univalent chromosomes per cell in those showing nonconjunction ranged from 1 to 8 in Marquillo and from 1 to 4 in Marquis. The failure of homologous chromosomes to conjugate, for some unknown reason, would account for those cases where the number of univalents was even. If such were the correct explanation, 40.7 per cent of those cells of Marquillo showing nonconjunction would be accounted for and 19.9 per cent of those of Marquis. The possibility that these univalents may be due to very weak synapsis followed by disjunction of the homologous chromosomes before or during early metaphase must not be overlooked. This will be discussed further in connection with plant culture 225-5 of Marquis which showed 40.2 per cent of nonconjunction.

Even though 40.7 per cent of the cases of nonconjunction in Marquillo and 19.9 per cent of those of Marquis can be accounted for by the above explanation, there still remains 59.3 per cent and 80.1 per cent of the cases to be explained. It seems that the phenomenon of polyvalence may be responsible for a part of the nonconjunction found. Plate 1, K-L, shows photomicrographs of trivalents, and it is believed that six chromosomes are involved in the conjugation shown in Plate 2, A. It was impossible to determine exactly how many chromosomes were united in the polyvalence shown in Plate 2, B. It seems that three chromosomes are joined end to end in the microsporocyte illustrated in Plate 2, C. The joined chromosomes extended over and beyond the equatorial plane and curved back again, the lower part not being in focus. Two other cases of trivalents of the type illustrated in Plate 1, K, were clearly distinguishable in this cell and there were three univalents off the equatorial plane. These cases of trivalents occurring in one cell and accompanied by univalents were found in a 42-chromosome plant. All the other cases illustrated were found in 41-chromosome plants. The microsporocyte illustrated by a photomicrograph in Plate 1, K, is shown as a camera-lucida drawing in Plate 3, F. There are two univalent chromosomes, as would be expected because of the trivalent association and because of the fact that this is a 41-chromosome plant. It should be added here that in the majority of the cells showing trivalence it was not possible to locate an accompanying single. However, this does not preclude the possibility of the single being present, as it might have been obscured in the main group of chromosomes, or it might have formed another polyvalent association. The frequency of the occurrence of trivalents and tetravalents for Marquillo and Marquis is given in Table 2. Only two cases of other polyvalent associations were found. In Marquillo, on an average, 1.4 per cent of the cells examined showed polyvalence, and 84.4 per cent of the number showing this phenomenon exhibited trivalents and the remaining 15.6 per cent tetravalents. In Marquis only 0.4 per cent of the pollen mother cells examined during metaphase revealed unions of more than two chromosomes. It does not seem that the amount of polyvalence exhibited by either Marquillo or Marquis is sufficient to account for 59.3 and 80.1 per cent, respectively, of cases of nonconjunction, which is the amount not accounted for by failure of homologous chromosomes to pair. Then it seems that either some of the univalents, as such, are hidden by the bivalents or else the

divided equationally is shown near the upper pole in Plate 2, I, whereas Plate 2, J, shows two chromosomes which have divided equationally. It seems highly probable that by dividing late nonoriented bivalents may give rise to the condition noted in Plate 1, G, and finally to micronuclei.

#### NONCONJUNCTION AND POLYVALENCE

From the foregoing results it seems that nonorientation of bivalents can account for some of the micronuclei occurring in the young microspores of Marquillo and Marquis. Other irregularities occur in the microsporogenesis of these two varieties and may be expected to have some influence on the prevalence of micronuclei. Univalents were found to be present, during metaphase of the first division, in some of the pollen mother cells of 42-chromosome plants of both Marquis and Marquillo. Evidently, for some reason or other, chromosomes which are usually conjugated at this stage of meiosis were univalent. This condition is termed "nonconjunction," and is illustrated in Plate 3, D, which is a camera-lucida drawing of a microsporocyte of Marquillo, culture 407-17-13. Frequently, cells having a single chromosome also showed three chromosomes united rather than the normal number of two (Pl. 1, K-L, pl 3, F). In still other cells higher numbers were involved in a single union. This phenomenon was termed polyvalence. Nonconjunction and polyvalence are discussed together because of the relationship that may exist between the two phenomena.

It is not difficult to distinguish between nonconjunction (pl 3, D) and predisjunction (pl 3, E) when the predisjoined members are on opposite sides of the equator. However, when they are both on the same side of the main group of dividing chromosomes they can be distinguished only by their orientation with reference to the achromatic figure, as is shown by a comparison of Plate 3, C and D, the former being considered a case of predisjunction. With the possible exception of plant 225-5 of Marquis, the phenomenon of the predisjoining members being on the same side of the equatorial plane was infrequent as compared with the occurrence of nonconjunction.

TABLE 2—Frequency, in percentage, of the occurrence of nonconjunction and polyvalence, and the number of univalents involved in microsporocytes of Marquillo and Marquis

Variety	Microspores showing—		Cells showing the indicated number of singles among those exhibiting nonconjunction						
	Nonconjunction	Polyvalence	1	2	3	4	5	6	8
Marquillo. ....	6.1±0.44	1.1±0.27	50.0	36.3	6.3	2.5	3.1	1.3	0.6
Marquis.....	7.7±.93	4±.10	78.3	18.8	1.8	1.1			

Table 2 gives the average percentage frequency of the occurrence of nonconjunction with the number of chromosomes involved and the average percentage frequency of polyvalence in the different cultures of Marquillo and Marquis.

An average of  $6.1 \pm 0.44$  of the microsporocytes of Marquillo showed nonconjunction and  $7.7 \pm 0.93$  per cent of those of Marquis. The difference between the two varieties is not statistically significant.

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polyvalent nature of some of the conjugants is obscured. The small correlation coefficient between polyvalence and nonconjunction of  $+0.28$  substantiates the conclusion that the amount of polyvalence discernible does not account for a large percentage of the cases of nonconjunction. According to Fisher's (2) table for small numbers, this correlation is not statistically significant. However, it should be noted that a high correlation could not be expected because it is not likely that all the cases of trivalents were discernible and also because a relatively high proportion of the nonconjunction noted probably was due to failure of homologous chromosomes to undergo synapsis.

Plant culture 225-5 of Marquis is of special interest because it exhibited nonconjunction in 40.2 per cent of the microsporocytes examined. E and F of Plate 1 are photomicrographs of pollen mother cells of culture 225-5. Plate 1, F, shows very weak conjugation between the bivalents not on the equatorial plane. Plate 1, E, shows a still greater separation between two single chromosomes that may have been paired. Of the 179 pollen mother cells examined in this plant 35 revealed one single chromosome, 31 two singles, 3 three singles, and 3 four singles. It seems as though in this particular plant two homologous chromosomes must have lost their affinity for each other, or perhaps that statement should be modified by saying the greater part of their affinity, as weak conjugation was noted in a number of cases. The occurrence of a high percentage of the microsporocytes of this plant showing nonconjunction might be expected to be reflected in a high percentage of the microspores showing micronuclei. Out of 552 immature pollen grains of other spikelets of the same plant examined, only 1.1 per cent showed micronuclei as compared to an average of 0.8 per cent for the 27 Marquis plants studied. Three hundred well-stained immature pollen grains from the same spikelet were studied and micronuclei were found in only 1.2 per cent of them. These results indicate that either the high percentage of nonconjunction noted is not common to all the microsporocytes of plant 225-5 or else the univalent chromosomes are reaching the poles during meiosis and being included in the micronuclei of the microspores. The former hypothesis seems the most plausible, as is shown in a report on culture 223, in a later section of this paper.

#### PREDISJUNCTION

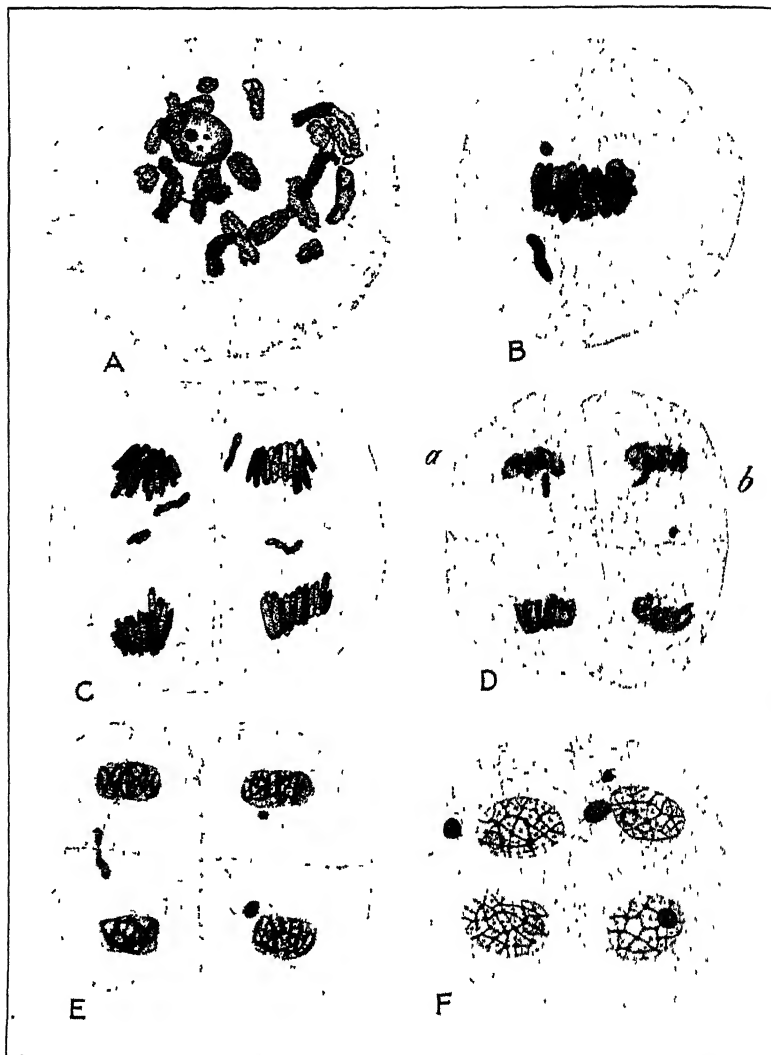
Only those cases in which both chromosomes were on opposite sides of the equatorial plane (pl. 3, B and E) were included in the determinations of the percentage of predisjunction. The microsporocytes of Marquillo showed  $6.3 \pm 0.69$  per cent of this phenomenon and those of Marquis  $2.8 \pm 0.31$  per cent. The difference between the two varieties is statistically significant, as can be seen from an examination of the probable errors. The range in Marquillo was from 0 to 18.5 per cent and in Marquis from 0 to 7.9 per cent. The number of bivalents involved in predisjunction ranged from 1 to 3 in both Marquillo and Marquis. It is difficult to determine the significance of predisjunction, but it seems doubtful whether this condition gives rise to micronuclei, as the chromosomes arriving at the poles early probably would be joined later by the main group.

## FRAGMENTATION

Irregularities other than those involving entire chromosomes were noted during microsporogenesis of Marquillo and Marquis. Plant 407-15-17, a 41-chromosome plant, showed during metaphase, in addition to the univalent chromosome, a small lagging fragment. It was possible to find the fragment of a chromosome not lined up on the equatorial plane in 70.2 per cent of the microsporocytes in the metaphase stage of the first division (Pl. 5, B). The fragment was not distinguishable in the remaining 29.8 per cent of the cells. It may have become obscured by having formed unions with other chromosomes or by having lined up on the equatorial plane. Plate 4, F, is an end view of the metaphase showing 20 pairs and 1 single, and offers some evidence for the hypothesis that the fragment may form unions with other chromosomes. The single chromosome appears constricted at one end, and as the fragment was not visible in this microsporocyte it seems plausible that the constriction might mark the point at which the fragment had become attached. A clearer case of an end constriction is shown in Plate 2, C, which is a photomicrograph of another plant of Marquillo.

Good preparations of microsporocytes of plant 407-15-17 in other stages of development were available also. The fragment was discernible in 83.9 per cent of the 93 cells examined in the diakinesis stage. It was usually located near the periphery of the nuclear cavity, as is shown in the upper portion of Plates 2, D, and 5, A, which are a photomicrograph and a camera-lucida drawing of the same cell. The presence of the fragment in the diakinesis and metaphase stages raises the question as to whether or not it can be found in the young microspores. Thirty-one and three-tenths per cent of the 198 microspores examined in the tetrad stage showed a small clump of chromatin (Pl. 5, D-F). In some instances (pl. 5, F) the small clump was accompanied by a larger micronucleus and in others by the macronucleus only. The distinction between the micronuclei and the small clumps of chromatin was made on the basis of size. Evidence that the two could be differentiated is obtained by comparing the percentage of microspores of other 41-chromosome plants showing micronuclei with that of plant 407-15-17. The percentage of microspores showing micronuclei in plant 407-15-17 was 30.3, and the average for six 41-chromosome plants was 23.4. The percentage for plant 407-15-17 is already somewhat higher than expected on the basis of other 41-chromosome plants and would be still higher if the percentage of microspores showing small clumps of chromatin in the cytoplasm were added to it. Thus, then, is further proof that the fragmented piece of chromosome seen in the diakinesis and metaphase stages is responsible for the small clumps of chromatin found in the microspore.

Second divisions were not available in plant 407-15-17, but plant 407-7-5 showed fragments in the second division (Pl. 2, K). Seventy-five microsporocytes in the metaphase of the second division were studied: 60 showed 1 fragment in each member of the diad, 6 showed 2 fragments in one member and none in the other, and 1 showed 2 fragments in each member. In the one case having 2 fragments in each member of the diad, the appearance in both is as if 1 may have divided to form 2. The position of the fragments



Stages of meiotic division, microsporocytes, camera-lucida drawings ( $\times 2,000$ )

- A — Fragment, above nucleolus, same cell as Plate 2, D, diakinesis of first division, 41-chromosome plant, Marquillo  
 B. — Univalent and fragment same plant as A, metaphase of first division, 41-chromosome plant, Marquillo  
 C — Lagging univalents, same plant as A and B, late anaphase of second division, 41-chromosome plant, Marquillo  
 D — Lagging chromosome (*a*) and fragment (*b*), same plant as A, B, and C, telophase of second division, 41-chromosome plant, Marquillo  
 E. — Fragmentation, fragment, and micronucleus, same plant as A, B, C, and D, telophase of second division, 41-chromosome plant, Marquillo  
 F — Fragment and two micronuclei, same plant as A, B, C, D, and E, tetrad, 41-chromosome plant, Marquillo

tion, together with its high frequency in the second division, indicates that in these plants the abnormality must have arisen in the somatic tissue or in the sexual cells of the preceding generation. If the latter supposition is correct, the behavior of the fragment may be likened to that of a univalent chromosome.

#### PROGENY OF SELECTED PLANTS

Progeny of three of the plants of Marquillo studied cytologically were grown in 1930 for the purpose of conducting further investigations. Culture 407-17-13 was grown because of the comparatively high percentage of the different aberrations found to occur during microsporogenesis. Three and five-tenths per cent of the microspores possessed micronuclei, 14.4 per cent of the microspores exhibited nonorientation, 18.8 per cent nonconjunction, and 16.0 per cent predisjunction. These are considerably above the averages for Marquillo, which were 2.8, 10.8, 16.1, and 6.3, respectively. In addition, the number of univalent chromosomes in the pollen mother cells showing nonconjunction varied from 1 to 8, the greatest range in any plant studied. The percentage of microsporocytes revealing polyvalence, was only 1 per cent above the average for Marquillo. Plate 3, B-E, shows camera-lucida drawings of aberrations found in the microspores of this plant. The progeny of culture 407-12-24 were included for further studies because, in general, its microsporocytes exhibited fewer aberrations than the average for all the plants of Marquillo. The regularity of the alinement of its chromosomes on the equatorial plane during metaphase of the first division is shown in Plate 1, B. Of the microspores examined, 2.6 per cent revealed micronuclei, and of the microsporocytes examined, 6.0 per cent showed nonorientation, 6.7 per cent nonconjunction, 4.4 per cent predisjunction, and 0 per cent polyvalence. Culture 407-12-3 of Marquillo was included because of its being a 41-chromosome plant.

The number of chromosomes in the 10 progeny of culture 407-17-13 was determined on the basis of the percentage of microspores showing micronuclei, with the exception of plants 223-10 and 223-4 the number of whose chromosomes was determined by counts made in the anaphase of the first division. That this is an accurate method of determining whether univalent chromosomes are present is shown by an examination of Table 3. As this table shows, the percentage of microspores of 41-chromosome plants exhibiting micronuclei varied only from 22.0 to 25.3 per cent. As all the plants of Marquillo studied had either the normal number of chromosomes, 21 pairs, or 20 pairs and 1 single, or 19 pairs and 2 singles, it seems safe to conclude that all the progeny of culture 407-17-13, with the exception of 223-10, had the normal number.

varied, being sometimes close to the equatorial plane and at other times some distance from it. A few telophases of the first division were available. Plate 2, I-J, shows photomicrographs of the same cell taken at different levels, and in order for the poles to correspond either the one or the other should be inverted. Both of the fragments were present and were in proximity to each other, appearing as though the two fragments had arisen from a division of one. Metaphases of the first division and microspores were not available.

It would be interesting to know the period or periods in the life cycle of the plant at which these fragments arise. It is not possible to say definitely at what stage fragmentation took place in the Marquillo cultures 407-15-17 and 407-7-5, but that fragmentation can take place during the anaphase of the first division is shown by Plate 2, F-H. The photomicrographs in Plate 2, F and G, are of the same microsporocyte taken at different levels. At the upper right side of the former is a chromosome, one of the chromatids of which reveals the beginning of a break. A chromosome in the lower right end of Plate 2, G, shows the same phenomenon in a more advanced condition, and Plate 2, H, represents a still more advanced stage with the fragmented piece at the equatorial plane. The chromatid involved seems to be breaking at approximately the center, as is shown by Plate 2, F. Camera-lucida drawings of the two microspores involved (pl 4, A and B) show the chromosome number of this plant (407-7-13) to be 42. These two cells were the only two microsporocytes of this plant exhibiting fragmentation. Also, 125 pollen mother cells in the metaphase stage of the first division were examined without evidence of fragmentation being found, proving that it occurred as noted for the first time during anaphase of the first divisions.

That fragmentation also occurs in Marquis is shown by Plate 2, E and L, the former being the metaphase of the first division and the latter the tetrad stage, showing a fragment in the cytoplasm of the upper left-hand microspore.

No definite attempt was made to determine the frequency of fragments in Marquillo and Marquis, but any fragmentation noted while the microsporocytes of these varieties were being studied for other abnormalities was recorded. Twelve of the 32 plants of Marquillo examined and 8 of the 27 plants of Marquis were recorded as showing some degree of fragmentation. From these figures it would seem that fragmentation is fairly common in both Marquillo and Marquis, but such a conclusion would be erroneous, as only one instance of fragmentation in any of the stages of a particular plant studied would have been recorded as the occurrence of fragmentation. That fragments could have been found in a relatively large proportion of the plants and still be of infrequent occurrence is emphasized by the fact that the total number of cells studied, including both microsporocytes and microspores, would average somewhat over 600 per plant.

Fragmentation has been shown to take place during the anaphase of the first division. This could not directly explain the occurrence of fragments in the diakinesis and metaphase stages of the first division of plant 407-15-17, nor could it be expected to explain the large proportion of the microsporocytes of plant 407-7-5, which showed this phenomenon during the metaphase of the second division. The fact that the diakinesis and metaphase stages show fragmenta-

Counts of plant 223-10, made from the anaphase of the first division, showed without exception that this plant had 41 chromosomes. This finding is further substantiated by an examination of microsporocytes in the metaphase of the first division and others in the tetrad stage. Of 119 microsporocytes in the metaphase of the first division 108 showed a univalent chromosome, 4 three univalents, and 7 no univalents. In 2 of the 7 microsporocytes not showing any single chromosomes two cases of trivalents such as noted in Plate 4, E, were clearly visible. These results show the chromosome behavior during the metaphase of the first meiotic division to be characteristic of the 41-chromosome plants already described and those to be discussed later. Twenty-one and seven-tenths per cent of the microspores in the tetrad stage exhibited micronuclei, which amount is very close to the 23.4 per cent exhibited by the 41-chromosome progeny of a 41-chromosome plant, culture 407-12-3. These results are important in showing that a 41-chromosome plant may arise from one having 42 chromosomes. From the foregoing discussion on chromosomal aberrations it is easy to visualize how such a 41-chromosome plant could originate. If a normal 21-chromosome male gamete fertilized a female gamete having only 20 chromosomes, owing to nonorientation or some of the other previously reported aberrations, a 41-chromosome zygote would result. As the spikes of this plant were not covered the previous year there still remains the possibility that the 41-chromosome plant might have resulted from pollination of a normal flower with stray pollen from another 41-chromosome plant. It can be shown from data given on 41-chromosome plants that the chances of the 41-chromosome plant being the result of chromosomal aberrations are fifty times greater than that of its being due to cross-pollination.

Another interesting abnormality was found in one of the progeny of culture 407-17-13. While making counts of the young microspores of plant 223-4 to determine the percentage that possessed micronuclei, it was noticed that the upper pollen sac of plant 223-4 was averaging strikingly higher in percentage of microspores showing one or more micronuclei than was the lower pollen sac of the same anther. It will be remembered that all spikelets were cut longitudinally and therefore would be expected to show a considerable number of microspores in each section. Counts were made in the four sections showing the two pollen sacs, the results obtained are recorded in Table 4.

TABLE 4.—*Variations between different pollen sacs of the same anther, in the percentage of microspores showing micronuclei, plant of Marquillo, culture No. 223-4*

Section No. *	Microspores in upper pollen sac			Microspores in lower pollen sac		
	Total	Showing micronuclei		Total	Showing micronuclei	
		Number	Per cent		Number	Per cent
1.....	56	11	19.6	42	0	0
2.....	68	22	32.4	54	1	1.9
3.....	98	17	17.3	65	3	4.6
4.....	75	15	20.0	41	0	0
Total.....	297	65		202	4	
Average.....			22.3±2.02			1.6±0.64

\*On slide No. 51.

TABLE 3—Chromosome number and behavior during meiosis of the progeny of a 41-chromosome plant of Marquillo, 407-12-3

Culture No	Number of chromosomes	Microspores showing indicated number of singles during metaphase						Total microspores	Microspores showing univalent		Tetravalent microspores*	Microspores showing		Total microspores	Microspores showing	
									Number	Per cent		Total	Per cent			
222-1*	40							120	100.0	4	0	4	3.3	462	106	42.4
222-3	41	5	71	102	5	8		272	93.8	3	1	4	5.2	537	131	24.4
222-5	41	11	218	1	24		1	263	98.8	2	0	2	1.9	644	163	25.3
222-6	41	30	180	1	5			192	90.6	2	0	2	1.4	564	124	22.0
222-7	41	16	48					64	75.0	2	0	2	3.1	318	114	22.0
222-8	41	32	234	2	7			243	88.4	6	1	7	5.1	460	105	22.8
222-9	41	4	138	2	3			153	97.5	6	2	8	5.1	475	113	23.8
Total		88	905	14	39		1	1,047	540.8	18	5	23	17.1	3,198	750	140.3
Percentage of total		8.4	86.4	1.3	3.7		1	100.0	90.1±2.15	78.3	21.7	100.0	2.9±5.0	333.0	125	23.4±2.4
Average		14.7	150.8	2.8	9.8			174.5								

<sup>a</sup> Omitted from calculations<sup>b</sup> Number of microspores showing some trivalent and tetravalent associations, respectively

86.4 per cent of the 1,047 microsporocytes examined showed 1 univalent chromosome. Also, 42.4 per cent of the microspores of plant 222-1 possessed micronuclei, as compared with  $23.4 \pm 0.24$  per cent for the 41-chromosome plants. These results support the counts showing plant 222-1 to have 40 somatic chromosomes, composed of 19 bivalents and 2 univalents. Table 3 shows that the data taken individually on the six remaining plants do not vary greatly, which is strong evidence supporting the counts that show these plants to be composed of 20 bivalent chromosomes plus 1 univalent.

These results are not those to be expected on the basis of random distribution of the single chromosome during meiosis of the microsporocytes and the megasporocytes. If the single chromosomes went at random to the poles during the first division, 50 per cent of the gametes would be expected to contain 21 chromosomes and the remaining 50 per cent would be expected to have 20 chromosomes. Random mating and survival of all the progeny would give a ratio of one 42-chromosome plant; two 41-chromosome plants; one 40-chromosome plant. Instead of this ratio six 41-chromosome plants and one 40-chromosome plant were obtained. The constitution of the 40-chromosome plants, according to theory, should be 20 bivalents and no univalents. The 40-chromosome plant was composed of 19 bivalents plus 2 singles, which makes it appear as though it should be classified among those having 41 chromosomes. The loss of a chromosome through nonorientation or some of the other aberrations noted in 42-chromosome plants would account for this condition of 19 pairs and 2 singles.

The behavior of the single chromosome during meiosis is important as its determination would furnish information as to the manner in which the different aberrations noted during metaphase of the first division of 42-chromosome plants give rise to micronuclei in the microspores. An attempt was made to follow the single chromosome through the various stages of meiosis. It was possible to distinguish the single chromosome in 86.4 per cent of the metaphases of the first division. Counts were made of the number of chromosomes at the poles in 27 microsporocytes in the anaphase of the first division. In 17 of these the single chromosome had gone to the poles without dividing or lagging, as was evidenced by there being 20 chromosomes at one pole and 21 at the other. In the other 10 cells the univalent chromosome was found lagging and dividing. Therefore, in 63 per cent of the cells the single chromosome was not lost, whereas in 37 per cent it was lost. On this basis 18.5 per cent of the microspores would be expected to show a micronucleus and the expected ratio of 20:21 chromosome gametes would be 69:31. The studies of the second division were made on the microsporocytes having both members of the diad present, and the percentage of microsporocytes showing the lagging chromosome was determined on the basis of both showing it. In the metaphase of the second division only 1 out of the 18 microsporocytes examined exhibited lagging chromosomes in both metaphases and none exhibited a lagging chromosome in only one member of the diad. On this basis only 2.7 per cent of the microspores would be expected to possess micronuclei. The results obtained can be explained by assuming that the already equationally divided chromosomes are included on the equatorial plane with the others. This agrees with the conclusions of Watkins (18) that the

In the upper pollen sac  $22.3 \pm 2.02$  per cent of the 297 microspores counted revealed micronuclei, whereas only 1.6 per cent of the microspores counted in the lower pollen sac exhibited this condition. The percentage of microspores showing micronuclei is very close to the average of  $23.4 \pm 0.24$  shown by 41-chromosome plants. This strongly suggests that the microsporocytes giving rise to the microspores must have possessed one unpaired chromosome, probably 20 bivalents plus 1 univalent. Since the lower pollen sac did not show this high percentage of abnormalities, the mutation giving rise to the univalent chromosome must have occurred some time during the development of the young anther, after the tissue giving rise to the pollen sacs had become differentiated, perhaps in the first archesporial cell. There were only 16 microsporocytes in the metaphase available, and only 1 showed a single chromosome. There were a number of microsporocytes in the diad stage, but lagging chromosomes were not any more frequent than in 42-chromosome plants of Marquillo. These results indicate that the high percentage of abnormalities is limited to the upper pollen sac of this one anther.

The average percentage of abnormalities in the microsporocytes of the progeny of culture 407-17-13 was  $4.7 \pm 1.44$ . The range in variability was from 0.5 per cent in plant 223-12 to 21.7 per cent in plant 223-10 and the coefficient of variability was 143.0.

The progeny of culture 407-12-24 was grown because in general this plant exhibited a lower percentage of the different aberrations than the average of the Marquillo plants studied. As determined by the percentage of microspores showing micronuclei, all of the 16 progeny studied possessed the normal number of chromosomes. It will be remembered that culture 407-12-24 exhibited micronuclei in 2.6 per cent of the microspores. The average percentage of the microspores of the progeny exhibiting micronuclei was  $2 \pm 0.13$ , and the range of variability was from 1.1 per cent to 3.4 per cent. The coefficient of variability was 39.7. These data show that the average percentage of micronuclei was only 0.6 of one per cent below that of the parent grown the previous year. Also, it is of interest to compare the progeny of culture 407-17-13 and 407-12-24. The difference of 2.7 per cent between the percentage of microspores showing micronuclei is not significant because of the high probable error of culture 407-17-13. The range of variability in progeny of culture 407-17-13 is 18.9 per cent higher than that for the progeny of culture 407-12-24. These data are based upon an examination of 5,610 microspores of the 10 progeny of culture 407-17-13 and 9,298 of the 16 progeny of culture 407-12-24.

Of the seven progeny of culture 407-12-3 studied, it was possible to obtain counts from the anaphase of the first division in all plants except 222-7. In this plant counts were made only from the metaphase of the first division. A summary of the data obtained is given in Table 3.

All plants were found to have 41 chromosomes, except 222-1, which possessed only 40, consisting of 19 pairs and 2 singles. Of the 120 microsporocytes of plant 222-1 examined, 102 showed two singles not on the equatorial plane, 5 one single, 5 three singles, and 8 four singles. These figures show that 85 per cent of the microsporocytes had 2 univalent chromosomes, which compares very favorably with the data obtained for the six 41-chromosome plants. In these plants

Generally it has been assumed by the various investigators that the reason for not being able to discern the univalent chromosome or chromosomes in all microsporocytes showing the metaphase of the first division is because of the other chromosomes obscuring it. The close agreement between the expected percentage of microspores showing micronuclei based on a study of the metaphase of the first division (Table 5) and that based on the anaphase or early telophase of the second division with the percentage actually found by counts made of the tetrad stage indicates that the univalent chromosome may not have been present in those cells in which it was not discernible. The extrusions of karyotin, previously described, would furnish the mechanism whereby it could have been eliminated during the early stages of sporogenesis.

The agronomic characters of the progeny of these cultures are reported in Tables 6 and 7.

Table 6 shows that Marquis surpassed culture 407-12-24 in number of spikes, percentage of fruitfulness, and height in inches, but was inferior in weight of seeds, percentage emerged based on number of seeds planted, and percentage matured based on the number of seeds planted. Marquis and culture 407-12-24 did not differ significantly in respect to their coefficients of variability. However, culture 407-12-24 had a higher coefficient of variability than Marquis as regards number of spikes and percentage of fruitfulness, but the reverse was true for weight of seed in grams and height in inches. Culture 407-17-13 without exception averaged higher in all six characters studied and in general showed a lower coefficient of variability. On the other hand, culture 407-12-3 averaged lower in all characters than any other culture studied with one exception. Marquis was lower in percentage of plants to emerge based on number planted. It will be remembered that culture 407-12-3 was a 41-chromosome plant. Culture 407-17-13 showed a high percentage of abnormalities during microsporogenesis, culture 407-12-24 exhibited a low percentage of abnormalities during microsporogenesis, and Marquis was planted for purposes of comparison. The progeny of the 41-chromosome plant possessed a higher coefficient of variability for every character studied. (Table 7)

TABLE 6—Average values of statistical measures of the characters of selected lines of Marquillo and Marquis from measurements made on individual plants

Variety or culture No.	Individuals	Height	Spikes	Fruitfulness	Weight of seed	Emergence based on number of seeds planted	Maturity based on number of seeds planted
	<i>Number</i>	<i>Inches</i>	<i>Number</i>	<i>Per cent</i>	<i>Grams</i>	<i>Per cent</i>	<i>Per cent</i>
Marquis.....	24	37 1±0.39	17 1±1 01	87 4±1 64	4 8±0 43	86 7	83 3
407-12-24.....	23	29 2± 17	13 4± 87	83 5±1 93	5 7± 48	92 0	84 0
407-17-13.....	15	34 4± 28	20 1±1 02	88 3±2 24	9 4± 74	100 0	93 8
407-12-3.....	7	25 6± 82	10 7±1 33	62 5±5 25	1 9± .33	88 9	80 0

single chromosomes dividing in the first division are regained in the second. Of the 51 pollen mother cells studied in the anaphase or early telophase of the second division, 22 exhibited lagging chromosomes in both members. On this basis 21.6 per cent of the microspores in the tetrad stage would be expected to show a micronucleus and the expected ratio of 20:21 chromosome gametes would be 72:28. A study was made on 3,198 microspores, 750 or 23.4 per cent of which showed a micronucleus. On this basis the expected ratio of 20:21 chromosome gametes would be 74:26. These data are summarized in Table 5.

TABLE 5—Percentage of microsporocytes showing the univalent chromosome lagging or off the equatorial plane during microsporogenesis of 41-chromosome plants

Stage of microsporogenesis	Microsporocytes		Expected percentage of microspores showing micronuclei	Expected ratio of 20:21 chromosome gametes
	Total	Showing univalents		
		Number	Per cent	
Metaphase of first division.....	1,047	905	86.4	21.8
Anaphase of first division.....	27	10	37.0	18.5
Metaphase of second division.....	18	1	5.6	2.7
Anaphase or early telophase of second division.....	51	22	43.1	21.6
Tetrad <sup>a</sup> .....	800	375	47.0	23.5
Back cross <sup>b</sup> .....				23.0
				73.27

<sup>a</sup> Counts made from examination of young microspores and converted to microsporocyte basis.

<sup>b</sup> Ratio of 20:21 chromosome gametes in megaspores as determined by back crossing to a 42-chromosome plant was 73.27 (Nishiyama 14).

Nishiyama (14) made a study of the ratio of 20:21 chromosome gametes by back crossing a 41-chromosome plant obtained from a cross of *Triticum polonicum* × *spelta* with *spelta*, and found that the ratio of 20:21 chromosome gametes was 73:27. He concluded from examination of Kihara's (11) and Watkins' (17) results that loss of chromosomes occurs with about the same frequency in microspore formation as in megaspore formation. If this assumption is correct, the results reported here are in very close agreement with those obtained by the back-cross method.

The data on the behavior of the univalent chromosome of 41-chromosome plants can be satisfactorily explained by the following hypothesis. The chances of the univalent chromosome undergoing the equational division in the first division are slightly less than those of its passing to the poles without dividing. In the latter case the univalent chromosome behaves normally and micronuclei are not formed in the microspores. In those cases in which the univalent chromosomes divide equationally in the first division the resulting daughter chromosomes are distributed to opposite dyads and both are included in the achromatic figures but are not discernible. However, another division does not occur but instead these chromosomes lag and eventually round up to form the micronuclei found in 23.4 per cent of the microspores of 41-chromosome plants. It should be noted here that the percentage of microspores showing micronuclei due to the univalent chromosome, is probably in reality somewhat lower than that reported because of other chromosomal aberrations producing the same phenomenon.

aberrations, nonorientation of bivalents, nonconjunction, and predisjunction played in causing the occurrence of the micronuclei found in microspores in the tetrad stage

TABLE 8—Coefficients of correlation between percentage frequencies of different aberrations found occurring during microsporogenesis of Marquillo and Marquis \*

Marquillo			Marquis		
Simple	Partial	N	Simple	Partial	N
$r_{12} + 0.70$	$r_{12\ 34} + 0.72$	30	$r_{12} + 0.70$	$r_{12\ 34} + 0.70$	27
$r_{13} + 0.32$	$r_{13\ 24} + 0.17$	25	$r_{13} + 0.17$	$r_{13\ 42} + 0.15$	26
$r_{14} + 0.21$	$r_{14\ 32} + 0.20$	27	$r_{14} + 0.14$	$r_{14\ 32} + 0.10$	25
$r_{23} + 0.12$	$r_{23\ 14} + 0.01$	25	$r_{23} + 0.12$	$r_{23\ 14} + 0.24$	26
$r_{24} - 0.05$	$r_{24\ 13} - 0.24$	27	$r_{24} - 0.25$	$r_{24\ 13} - 0.23$	25
$r_{34} + 0.58$	$r_{34\ 12} + 0.54$	24	$r_{34} - 0.31$	$r_{34\ 12} - 0.30$	25
$R_{1\ 2\ 3\ 4}$			71		

\* Key to numbers 1=micronuclei, 2=nonorientation, 3=nonconjunction, 4=predisjunction. Levels of significance according to Fisher's (2) tables for the values of the correlation coefficient for different levels of significance an  $N$  of 20 and  $r$  of 0.42 gives a  $P$  value of 0.05

The only significant simple and partial correlations obtained in both Marquis and Marquillo were those between nonorientation of bivalents and micronuclei, the simple correlation being +0.70 in both Marquillo and Marquis, and the partial correlations being +0.72 and +0.70, respectively. Both the simple and partial correlations between nonconjunction and micronuclei in Marquillo and Marquis were positive but not statistically significant, being +0.32 and +0.17, respectively, for Marquillo and +0.17 and +0.15 for Marquis. The simple correlations between nonorientation and nonconjunction in both Marquillo and Marquis were +0.12 and the partial correlations were +0.01 and -0.24, respectively. None of these values are statistically significant.

These results indicate that nonorientation and nonconjunction are independent of each other, and such a conclusion is what would be expected from the cytologic studies. Such is not true, however, of the association between nonconjunction and micronuclei, as there seems to be no cytological basis for assuming that at least a part of the univalents found during metaphase of the first division should not give rise to micronuclei in the tetrads. Especially is this true since the univalent chromosome of 41-chromosome plants has been found to do so. Then the question arises whether the correlations found measure the true relationship existing between nonconjunction and micronuclei even though they are not statistically significant. The fact that all the correlations between these two aberrations in both Marquillo and Marquis are positive indicates that they are probably more important than the level of significance set up indicates. Therefore, considering both the cytologic studies and the coefficients of correlation it seems that the phenomenon of nonconjunction gives rise to micronuclei.

In the studies with Marquillo a simple correlation of +0.21 and a partial correlation of +0.20 were obtained between micronuclei and predisjunction. The corresponding correlations in Marquis were -0.14 and +0.10. The simple and partial correlations between nonconjunction and predisjunction were +0.58 and +0.54, respectively, in Marquillo and -0.31 and -0.30 in Marquis. The correla-

TABLE 7—Coefficients of variability of characters of the progeny of selected lines of Marquillo and Marquis from measurements made on individual plants

Variety or culture No	Individuals	Height	Spikes	Fruitfulness	Weight of seed
	Number	Inches	Number	Per cent	Grams
Marquis.....	24	7 7±0 75	42 7± 4 86	13 7±1 36	65 8± 8 75
407-12-24.....	23	4 1± 41	46 0± 5 46	16 4±1 67	59 6± 7 75
407-17-13.....	15	4 6± 57	29 1± 3 87	11 5±1 82	45 3± 6 62
407-12-3.....	7	12 5±2 29	48 9±10 72	33 0±6 56	68 9±17 31

## CORRELATIONS BETWEEN CYTOLOGIC ABERRATIONS

It seemed important to determine the relationships of the different aberrations to each other and if possible the influence that they may exert upon the characters of the plant. Since it was impossible to distinguish qualitative differences, these characters have been omitted from the discussion. All measurements of quantitative characters were made on individual plants and expressed on the plant basis. It is important to keep in mind that the correlations between abnormalities and plant characters calculated for Marquillo, with the exception of one correlation on fruitfulness, are not comparable with those of Marquis, the reason for this being that the characters correlated with aberrations in Marquillo are of the progeny of the plants studied cytologically, whereas those of Marquis are the characters of the plants studied cytologically. This would make the two sets of data differ in two essential points. (1) The microsporocytes studied give rise to the plants with whose characters their abnormalities are correlated and (2) the measurements are based on the average of many plants in Marquillo. In the case of Marquis the abnormalities of the microsporocytes are correlated with the characters of the plants producing them and of necessity the measurements are based on only one plant. However, the correlations between the different abnormalities found in Marquillo and the same correlations for Marquis are comparable.

For determining the statistical significance of coefficients of correlation, Fisher's (2) table giving values of the correlation coefficient for different levels of significance was used. Coefficients of correlation giving a  $P$  value of 0.05 were considered as being statistically significant. If  $N$  is 20 a correlation of 0.42 would be necessary to give a  $P$  value as low as 0.05. Fisher's tables have been used because the coefficients of correlation reported in this work are based upon small numbers. It is usual to measure the significance of the correlation  $r$  by employing the probable error obtained by the formula  $Er = 0.6745 \frac{1-r^2}{\sqrt{n}}$ . With small samples the value of  $r$  is often very

different from the true value and consequently  $1-r^2$  is in error, and also the distribution of  $r$  for high correlations in small samples is far from normal, so that tests of significance based on the  $Er$  determined from this formula may be misleading. For practical purposes by the use of Fisher's table these two errors are avoided.

Table 8 gives the simple and partial correlations obtained between percentage frequency of different aberrations occurring during microsporogenesis of Marquillo and Marquis. These correlations were calculated in an endeavor to determine the part that each of the

Progeny of Marquillo plants studied cytologically in 1929 were grown in 1930, and statistical measures were taken of percentage of plants emerged based upon number of seeds planted, percentage of plants matured based upon number of seeds planted, number of spikes per plant, weight of seed per plant, height of individual plants, and fruitfulness of outside florets of noncovered spikes. Coefficients of variability were calculated for these last four characters and then correlated with the percentage frequency of the different cytologic aberrations found in the parents grown in 1929.

The results of the studies on the coefficients of correlation between percentage frequencies of different aberrations found occurring during microsporogenesis of Marquillo and coefficients of variability of characters of the progeny are given in Table 10. Neither micronuclei, nonorientation, nor nonconjunction showed significant simple or partial correlations with the coefficient of variability of number of spikes per plant. Micronuclei with the coefficient of variability of weight of seed per plant gave a coefficient of correlation of +0.51, with nonconjunction a correlation of +0.45, and with nonorientation a correlation of +0.33. The first two are higher than +0.42, which is taken as the level of significance, and the latter lacks only +0.09 of being significant. Micronuclei and nonorientation gave significant correlations with both the coefficient of variability of height of plant and fruitfulness, being +0.47 and +0.53, respectively, in the former correlation and +0.61 and +0.49 in the latter. Nonconjunction with the coefficient of variability of these two characters gave correlations of +0.11 and +0.27, respectively. None of the partial correlations were statistically significant according to the standards set up, but all of the 12 were positive with the exception of the correlation between the coefficient of variability of number of spikes per plant and micronuclei. All of the multiple correlations were significant with the exception of number of spikes per plant correlated with micronuclei, nonorientation, and nonconjunction. These results rather definitely show that there is a positive relationship between cytologic aberrations and the coefficients of variability of the three plant characters, weight of seed, height of plants, and fruitfulness. Micronuclei and nonconjunction are more highly correlated with the coefficients of variability of weight of seed per plant than is nonorientation, and micronuclei and nonorientation are more highly correlated with the coefficients of variability of height of plants and fruitfulness than is nonconjunction. These results indicate that the micronuclei, nonorientation, and nonconjunction are causing variability in the three plant characters mentioned above or else are associated with some factor or factors causing variability.

tions between nonconjunction and predisjunction are statistically significant in Marquillo but are not in Marquis. It seems doubtful whether predisjunction is instrumental in the production of micronuclei in the tetrads. This is in agreement with the cytologic evidence.

From a consideration of all available data it seems logical to conclude that nonorientation is most prominent in the production of micronuclei, but that nonconjunction also plays some part.

#### CORRELATIONS BETWEEN PERCENTAGE FREQUENCY OF DIFFERENT ABERRATIONS AND STATISTICAL MEASURES OF PLANT CHARACTERS

The coefficients of correlation were used to determine the relationship existing between the abnormalities occurring during microsporogenesis and plant characters. The data may be separated into two distinct groups. In the first group are included the coefficients of correlation between cytologic aberrations and the statistical measure of characters of the plants which produced them, and in the second group are given the coefficients of correlation between cytologic abnormalities and the statistical measures of the characters of the progeny of the plants in which these aberrations were studied. Only quantitative characters were used in the studies as no differential qualitative characters were found.

The only plant character obtained in Marquillo plants examined cytologically was percentage of fruitfulness. The correlations of fruitfulness with micronuclei, nonorientation, and nonconjunction were +0.19, +0.22, and -0.06, respectively. These results are not statistically significant.

Table 9 gives the simple, partial, and multiple correlations for Marquis. Statistically none of the correlations are significant, but it is worthy of note that of the 12 simple coefficients of correlation calculated all but two are negative and these two are very low, being +0.06 and +0.10. The highest correlations were obtained between the cytologic aberrations and weight of seed, and in every case they were negative. These results indicate that there may be some correlation between cytologic aberrations and the characters of the plant in which they occur.

TABLE 9—Coefficients of correlation between percentage frequencies of different aberrations found occurring during microsporogenesis of Marquis and statistical measures of characters of this variety \*

	Simple	Partial
Number of spikes per plant (5)-----	$r_{51} = -0.22$ $r_{52} = -0.05$ $r_{53} = -0.06$ $R_{5\ 123} = .37$	$r_{51\ 23} = -0.36$ $r_{52\ 13} = .29$ $r_{53\ 12} = .11$
Weight of seed per plant (6)-----	$r_{61} = -.23$ $r_{62} = -.12$ $r_{63} = -.14$ $R_{6\ 123} = .26$	$r_{61\ 23} = .20$ $r_{62\ 13} = .06$ $r_{63\ 12} = .10$
Height of plants (7)-----	$r_{71} = .15$ $r_{72} = .10$ $r_{73} = .14$ $R_{7\ 123} = .34$	$r_{71\ 23} = .29$ $r_{72\ 13} = .29$ $r_{73\ 12} = .12$
Fruitfulness of outside florets (8)-----	$r_{81} = .16$ $r_{82} = .25$ $r_{83} = .04$ $R_{8\ 123} = .25$	$r_{81\ 23} = .03$ $r_{82\ 13} = .20$ $r_{83\ 12} = .02$

\* Key to numbers: 1=micronuclei, 2=nonorientation, 3=nonconjunction. N 24. Levels of significance according to Fisher's (2) tables for the value of the correlation coefficient for different levels of significance an N of 20 and an  $r$  of 0.42 gives a  $P$  value of 0.05.

TABLE 11 — *Coefficients of correlation between percentage frequencies of different aberrations found occurring during microsporogenesis of plants of Marquillo and characters of these plants*<sup>a</sup>

Characters	Simple	Partial
Average number spikes per plant (5) -----	$r_{51} - 0.16$ $r_{52} - 0.05$ $r_{53} - 0.34$ $R_5^{123} - 0.35$	$r_{51} 23 - 0.07$ $r_{52} 13 + 0.04$ $r_{53} 12 - 0.31$
Average weight of seed per plant (6) -----	$r_{61} - 0.38$ $r_{62} - 0.11$ $r_{63} - 0.34$ $R_6^{123} - 0.48$	$r_{61} 23 - 0.35$ $r_{62} 13 + 0.20$ $r_{63} 12 - 0.23$
Average height of plants (7) -----	$r_{71} - 0.19$ $r_{72} - 0.25$ $r_{73} - 0.38$ $R_7^{123} - 0.45$	$r_{71} 23 + 0.11$ $r_{72} 13 - 0.24$ $r_{73} 12 - 0.38$
Percentage fruitfulness of outside florets of noncovered spikes (8) -----	$r_{81} - 0.48$ $r_{82} - 0.42$ $r_{83} - 0.35$ $R_8^{123} - 0.54$	$r_{81} 23 - 0.20$ $r_{82} 13 - 0.18$ $r_{83} 12 - 0.26$
Percentage emergence based upon number of seeds planted (9) -----	$r_{91} + 0.02$ $r_{92} - 0.09$ $r_{93} - 0.45$ $R_9^{123} - 0.51$	$r_{91} 23 + 0.31$ $r_{92} 13 - 0.25$ $r_{93} 12 - 0.51$
Percentage matured based upon number of seeds planted (X) -----	$r_{X1} + 0.04$ $r_{X2} + 0.05$ $r_{X3} - 0.46$ $R_X^{123} - 0.50$	$r_{X1} 23 + 0.20$ $r_{X2} 13 - 0.06$ $r_{X3} 12 - 0.50$

<sup>a</sup> Key to numbers, 1=micronuclei, 2=nonorientation, 3=nonconjunction. N=23. Levels of significance. According to Fischer's (2) tables for the values of the correlation coefficient for different levels of significance an N of 20 and an  $r$  of 0.42 gives a  $P$  value of 0.05.

In summarizing it may be said that some one, or in some cases maybe all, of the cytologic abnormalities are negatively correlated with the plant characters studied except the plant character average number of spikes per plant. Micronuclei, nonorientation, and nonconjunction seem to be negatively associated with average weight of seed per plant, average height of plants, and percentage of fruitfulness, and probably nonconjunction alone is negatively associated with percentage emergence and percentage matured.

#### PERCENTAGE OF NATURAL CROSSING IN MARQUILLO

It is of interest to determine the amount of natural crossing in Marquillo, since Hollingshead (7) has found a high percentage of nonconjunction in some  $F_1$  hybrids, especially when Garnet and Marquillo were the parents. That natural crossing in Marquillo is probably frequent has been well recognized by workers at the Minnesota Agricultural Experiment Station. Goulden and Neatby (3) found this to be true, and in addition they found that the amount of natural crossing varied between lines and between different years. Hayes (5) and Hayes and Garber (6) report natural crossing in wheat to vary from 2 to 3 per cent at University Farm, St. Paul, Minn.

It will be remembered from the discussion under Materials and Methods that seeds from both covered and noncovered heads were tested for susceptibility to black stem rust, form 21. Figure 1 shows the difference in susceptibility among plants. The six leaves at A are from plants grown from seed of covered spikes and show different degrees of resistance. The nine leaves at B were grown from seed of noncovered spikes, the first six on the left showing variation in degrees of resistance and the last three on the left showing susceptibility. Once they had become mixed, it would be impossible to isolate these from the susceptible leaves taken from plants of Ceres shown at C. Then, as this picture shows, it was very easy to separate the susceptible and resistant plants.

TABLE 10—Coefficients of correlation between percentage frequencies of different aberrations found occurring during microsporogenesis of plants of Marquillo and coefficients of variability of characters of progeny of these plants <sup>a</sup>

Characters	Simple	Partial
Number of spikes per plant (5).....	$r_{51} -0.37$ $r_{52} -21$ $r_{53} +02$ $R_{5\ 123} 40$	$r_{51\ 23} -0.33$ $r_{52\ 13} +02$ $r_{53\ 12} +17$
Weight of seed per plant (6).....	$r_{61} +51$ $r_{62} +35$ $r_{63} +15$ $R_{6\ 123} 60$	$r_{61\ 23} +32$ $r_{62\ 13} +01$ $r_{63\ 12} +35$
Height of plants (7).....	$r_{71} +47$ $r_{72} +53$ $r_{73} +11$ $R_{7\ 123} 55$	$r_{71\ 23} +15$ $r_{72\ 13} +32$ $r_{73\ 12} +01$
Fruitfulness of outside florets of noncovered spikes (8).....	$r_{81} +61$ $r_{82} +49$ $r_{83} +27$ $R_{8\ 123} 62$	$r_{81\ 23} +38$ $r_{82\ 13} +13$ $r_{83\ 12} +12$

<sup>a</sup> Key to numbers 1=micronuclei, 2=nonorientation, 3=nonconjunction. N=23. Levels of significance. According to Fisher's (2) tables for the values of the correlation coefficient for different levels of significance an N of 20 and an  $r$  of 0.42 gives a  $P$  value of 0.05.

Table 11 gives the coefficients of correlation between percentage frequencies of different aberrations occurring during microsporogenesis of plants of Marquillo and values of statistical measurements of characters of their progeny. The aberrations used in the studies were micronuclei, nonorientation, and nonconjunction, and the characters were average number of spikes per plant, average weight of seed per plant, average percentage of fruitfulness, percentage emergence based upon number of seeds planted, and percentage matured based upon the number of seeds planted. None of the correlations with average number of spikes per plant were significant. Only four statistically significant simple correlations were obtained. Micronuclei and nonorientation gave a  $-0.48$  and  $-0.42$ , respectively, with percentage of fruitfulness, and nonconjunction gave correlations of  $-0.45$  and  $-0.46$ , respectively, with percentage emerged and percentage matured. The only two significant partial correlations of  $-0.51$  and  $-0.50$  were obtained between percentage matured and nonconjunction, holding micronuclei and nonorientation constant, and percentage matured and nonconjunction, holding the same two variables as before constant. None of the multiple correlations in which the plant character was correlated with the three chromosomal aberrations was statistically significant. None of the simple correlations between average weight of seed per plant and any of the cytologic abnormalities was statistically significant, nor were the simple correlations statistically significant between average height of plants and any of the cytologic aberrations, micronuclei, nonorientation, or nonconjunction. However, that a true correlation might exist between these two plant characters and chromosomal aberrations seems evident from the fact that the correlations obtained were negative in all cases.

## DISCUSSION

The question whether the desirable qualities of the emmer group of wheats can be combined with those of the *vulgare* group into a germinally stable variety is highly important to anyone endeavoring to improve this crop. Marquillo, Marquis, and Minnesota 2303 were used in the investigation of this problem. Marquillo was produced from a cross of Marquis with a highly rust-resistant variety of durum called Tumillo, and it combined the stem-rust resistance of the durum parents with desirable characters of the *vulgare* parent. However, under field conditions it proved to be more variable in agronomic characters than Marquis or other common wheat varieties and yielded a flour which produced bread of somewhat inferior color but satisfactory in every other particular. Marquis is grown more widely in the hard red spring wheat areas of the United States and Canada than any other variety. It is an excellent milling and baking wheat, in general possesses desirable agronomic characters, and produces fair yields except when damaged by black stem rust. Marquillo in the field is resistant to black stem rust and therefore represents a distinct step forward in combining the desirable characters of the durum and *vulgare* wheats into a single variety. Realizing the desirability of continuing beyond the progress already made, investigators at the University of Minnesota, cooperating with the Division of Cereal Crops and Diseases of the United States Department of Agriculture, crossed a sister selection of Marquillo with Kanred  $\times$  Marquis selections which excelled in agronomic characters. From this cross was derived Minnesota 2303, which experiments to date have shown to be fully equal to Marquis in milling and baking qualities, including color of bread produced from the flour; superior in yielding ability, fully equal in other agronomic characters, and in addition to possess resistance to black stem rust. Minnesota 2303 combines the desirable characters of the durum and *vulgare* wheat groups. Since Marquillo exhibited more variability of agronomic characters than other common varieties, and Minnesota 2303 gives promise of becoming a highly desirable economic variety, it seemed advisable to determine their germinal stability in comparison with Marquis as evidenced by cytologic studies.

Minnesota 2303 was included in the studies on the frequency of the occurrence of micronuclei only. The results reported in this paper show that Minnesota 2303 in respect to the chromosomal aberration is no more unstable germinally than Marquis. Later unpublished work including nonorientation and nonconjunction confirmed this conclusion based upon a study of the occurrence of micronuclei. Even though Marquillo was found to be more unstable germinally than Marquis and this instability was shown to be associated with agronomic variability, the data obtained from studies with Minnesota 2303 prove that the desirable characters of the durum and common wheat groups can be combined in a germinally stable variety.

The fact that germinal instability is associated with variability of agronomic characters makes it important to determine whether this phenomenon is due to natural crossing which would produce variability and the correlations noted. This does not seem probable as the 7.2 per cent of natural crossing found in Marquillo would produce only approximately 2 hybrid plants among the 23 whose

The results obtained are reported in Table 12. Plants grown from seed of covered spikes varied in percentage of susceptible plants from 0 to 12, depending upon the line, while the different lines grown from seed of noncovered spikes varied in susceptibility from 0 to 9.8 per cent and had an average of  $3.6 \pm 0.50$  per cent of susceptible plants. The lines grown from seed of covered spikes showed an average of  $0.4 \pm 0.11$  per cent of susceptible plants, and all the plants of Ceres grown as checks proved to be susceptible. The chances of the difference of  $3.2 \pm 0.51$  per cent between covered and noncovered seed being due to the probable errors of random sampling are very small. Griffie and Hayes (4) have pointed out that the percentage of plants showing evidence of crossing should be doubled in order to obtain a true measure of the amount of natural crossing that takes place. On this basis, if the percentage of susceptible plants can be used as

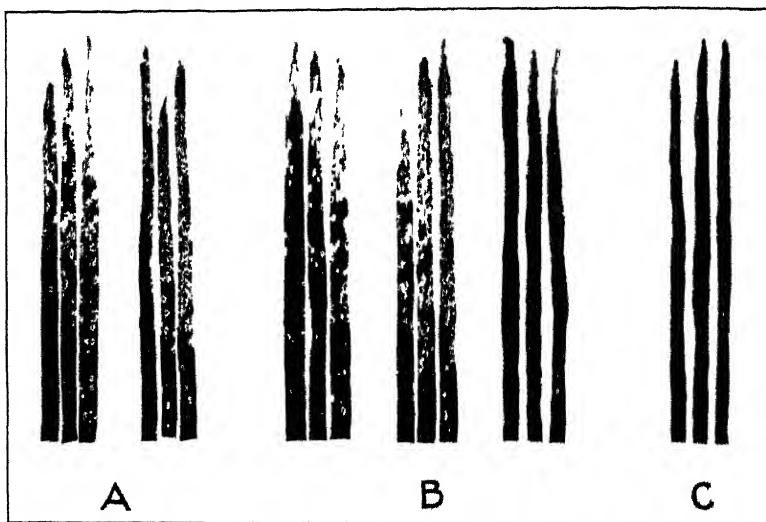


FIGURE 1—Reaction of seedlings of Marquillo, Ceres, and natural hybrids between these two varieties to black stem rust, form 21. A, Leaves of seedlings grown from seed of covered spikes of Marquillo, B, leaves of seedlings grown from seed of noncovered spikes of Marquillo, the three at the right being natural hybrids, C, leaves of seedlings grown from seeds of Ceres.

a measure of natural crossing, the amount occurring in Marquillo would be 7.2 per cent.

TABLE 12—Percentage of susceptible seedlings in different lines of Marquillo

Culture No	Plants	Seedlings from covered spikes			Seedlings from noncovered spikes		
		Total	Susceptible		Total	Susceptible	
	Number	Number	Number	Per cent	Number	Number	Per cent
407-12-20.....	19	127	0	0	214	21	9.8
407-9-3.....	14	114	1	0.9	169	10	5.9
407-3-417.....	24	243	3	1.2	371	16	4.6
313-54.....	21	148	0	0	245	8	3.3
407-17-12.....	13	104	0	0	151	4	2.6
410-55.....	14	164	1	.6	212	3	1.4
407-19-13.....	13	123	0	0	175	2	1.1
371-6.....	12	126	1	.8	144	0	0
Average.....				$0.4 \pm 0.11$			$3.6 \pm 0.50$

\* Difference between those two is  $3.2 \pm 0.51$

(Pls 2, A, and 3, F.) Predisjunction is used to denote the disjunction of a bivalent or bivalents in advance of the main group of conjugated chromosomes during metaphase of the reductional division (Pl 3, E)

Two of the 32 plants of Marquillo were found to have 41 somatic chromosomes, those remaining having the normal number of 42, and all of the plants of Marquis and Minnesota 2303 possessed the normal chromosome number

Two and eight-tenths per cent of the microspores of Marquillo revealed micronuclei, while 0.8 per cent each of Marquis and Minnesota 2303 showed similar aberrations

Extrusion of karyotin as shown in Plate 1, A, was found in 2.8 per cent of the very young microsporocytes of Marquillo, culture 407-15-17

Nonorientation of bivalents was found to occur in  $10.8 \pm 0.68$  per cent of the cells of the young microsporocytes of Marquillo and in  $6.9 \pm 0.49$  per cent of those of Marquis

Nonconjunction was exhibited in the metaphase of the first division by  $6.1 \pm 0.44$  per cent of the Marquillo microsporocytes and in  $7.7 \pm 0.93$  of those of Marquis

In Marquillo on an average 1.4 per cent of the cells examined showed polyvalence, whereas 0.4 per cent of the pollen mother cells of Marquis were found to show this condition.

It does not seem that the amount of polyvalence exhibited by either Marquillo or Marquis is sufficient to account for 59.3 and 80.1 per cent, respectively, of cases of nonconjunction, which is the amount that could not be accounted for by failure of homologous chromosomes to pair

The microsporocytes of Marquillo showed  $6.3 \pm 0.69$  per cent of predisjunction and those of Marquis  $2.8 \pm 0.31$  per cent

Probably both nonorientation of bivalents and nonconjunction give rise to micronuclei found in the microspores, but it is doubtful whether predisjunction causes this phenomenon.

Microsporocytes showing fragments of chromosomes were found in both Marquillo and Marquis

The progeny of culture 407-17-13 were grown for further cytological studies because of the high percentage of abnormalities exhibited by this plant. One of the progeny was found to possess 41 somatic chromosomes. In another progeny of this plant was found an anther in which the two pollen sacs studies exhibited differences in the percentage of microspores possessing micronuclei. In the upper pollen sac  $22.3 \pm 2.02$  per cent of the microspores possessed micronuclei, whereas only  $1.6 \pm 0.64$  per cent of the microspores counted in the lower pollen sac exhibited this condition.

The progeny of culture 407-12-24 were grown because the percentage of chromosomal aberrations exhibited by this plant was low. All the progeny were found to have 42 somatic chromosomes and the percentage of abnormalities was again low.

The progeny of culture 407-12-3 were grown because this plant possessed only 41 somatic chromosomes. One of the progeny was found to have 19 bivalents plus 2 univalents, whereas the other 6 possessed 41 somatic chromosomes.

An average of  $23.4 \pm 0.24$  per cent of the microspores of the 41-chromosome plants showed micronuclei. On the basis of the num-

progeny were grown for the purpose of correlating cytologic aberrations found in the parents with variability and average values of agronomic characters of the progeny. That none of these 23 plants were hybrids was indicated by the fact that it was impossible to find them segregating for any qualitative characters. Even if two hybrid lines, the number expected due to natural crossing, were present among the 23 lines studied it is evident that they could hardly have been responsible for the correlations previously reported. Moreover, it does not seem that natural crossing can explain all of the variations in percentage of chromosomal aberrations found occurring among the different plants of Marquillo and Marquis, for if natural crossing were instrumental in causing high frequencies of aberrations, these different aberrations should be positively correlated. Nonorientation did not show a significant correlation with either nonconjunction or predisjunction. Therefore, it seems that natural crossing is not responsible for the germinal instability found in Marquillo and consequently it may be concluded that germinal instability is directly responsible for at least some of the variability within agronomic characters of this variety.

These data emphasize the importance of cytology as an aid to plant breeding. More work needs to be done before the exact importance of the different chromosomal aberrations can be definitely determined. However, the data available are strongly indicative of the importance of chromosomal aberrations in producing certain phenomena pertinent to the problems which arise in any crop improvement program and consequently these phenomena should be given consideration. A statistically significant negative correlation was obtained between chromosomal aberrations and percentage fruitfulness, indicating that these aberrations may be causing sterility in some of the florets. Leighty and Taylor (12) and Goulden and Neatby (3) point out that natural crossing seems to be associated with sterility. If such is the case, it seems that chromosomal aberrations may be the indirect cause of the large percentage of natural crossing that Leighty and Taylor (12) reported for some varieties. Also, variations due to cytologic anomalies may be expected to behave similarly to environmental variations, and it is doubtful whether they could be eliminated by selection. For example, it was impossible to identify the 41-chromosome plants on the basis of agronomic characters.

#### SUMMARY

Thirty-two plants of Marquillo, 27 plants of Marquis, and 30 plants of Minnesota 2303 were studied cytologically. However, the latter variety was included only in the studies on the percentage of microspores showing micronuclei. The use of certain terms is explained. Nonorientation of bivalents has been applied to the occurrence of a bivalent or bivalents off the equatorial plane just prior to the disjunction of the main group of bivalents during metaphase of sporogenesis (Pl. 1, C and D). Nonconjunction is used to signify the occurrence of a univalent or univalent chromosomes during metaphase of the reductional division, when presumably the homologous mates of this univalent or these univalent chromosomes are present. (Pl. 3, A). Polyvalence has been employed to designate the union of three or more chromosomes during the metaphase of the reductional division.

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ber of microspores showing micronuclei the expected ratio of 20:21 chromosome gametes was found to be 74:26 Nishiyama (14) by using the back-cross method determined the expected ratio of 20:21 chromosome gametes to be 73:27

The results obtained with these 42-chromosome plants strongly indicate that in somewhat more than 50 per cent of the microspores in the anaphase of the first division the univalent chromosome went to the poles without dividing. The chromosomes resulting from those cases in which the univalent divided, did not divide in the second division but lagged and eventually formed the micronuclei occurring in the tetrads.

By determining the percentage of microspores showing micronuclei and making the necessary calculations, the frequency of plants having abnormal chromosome numbers may be predicted for any variety.

The coefficients of correlation calculated between percentage frequencies of different aberrations indicate that micronuclei are associated with both nonorientation of bivalents and nonconjunction, but that no correlation exists between nonorientation and nonconjunction

The coefficients of variability of the characters of the progeny of the Marquillo plants studied cytologically were calculated and then correlated with percentage frequency of the different chromosomal aberrations. Statistically significant positive simple correlations were found between the coefficient of variability of weight of seed per plant and micronuclei as well as nonconjunction; coefficient of variability of height of plants and micronuclei as well as nonorientation of bivalents, and coefficient of variability of fruitfulness and micronuclei as well as nonorientation of bivalents

Likewise, the average values of the characters of the progeny of the Marquillo plants studied cytologically were calculated and then correlated with percentage frequency of the different chromosomal aberrations. Statistically significant negative correlations were obtained between percentage fruitfulness and micronuclei as well as nonorientation of bivalents, between percentage emergence and nonconjunction, and between percentage of seeds which produced mature plants and nonconjunction.

The percentage of natural crossing in Marquillo was found to be 7.2, which is higher than has been generally found for other varieties at the Minnesota station

Marquillo was found to possess greater germinal instability than Marquis, but Minnesota 2303 was found to be the equal of Marquis in germinal stability, thus proving that the desirable qualities of the durum and *vulgare* wheats can be combined into a single germinally stable variety

The germinal instability of Marquillo was found to be associated with the greater variability of agronomic characters possessed by this variety.

The data indicate that germinal instability is responsible for the high percentage of natural crossing found in Marquillo.

Variations due to germinal instability of varieties can be distinguished from environmental variations only by combined cytologic and genetic studies

# SOME EFFECTS OF ROOT ROT ON THE PHYSIOLOGY OF PEAS<sup>1</sup>

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## INTRODUCTION

The disease complex known as root rot of peas (*Pisum sativum* L.) is a disturbing element in the production of the high quality in peas necessary for a Fancy canned or market product. Canners' field men have long known that in a pea field spotted with yellow-root-rot-affected areas it is difficult to determine the time to harvest the crop for maximum quality. The yellowed plants pass more rapidly up to and beyond the canning stage than the more nearly normal plants in the same field. If the field is harvested when the peas on the diseased vines reach the canning stage, a considerable yield increment that would come from the more slowly developing healthy vines is sacrificed. If, on the other hand, harvest is delayed for the yield increment from the healthy vines, the uniformity and quality of the pea pack are reduced.

Root rot occurs in scattered spots over a field of peas. The below-ground parts of affected plants turn dark, die, and rot; and the leaves become yellow and die progressively from the bottom of the plant upward. The entire diseased plant from the lowest leaf to the tip of the stem, including the pods, is a lighter shade of green than a healthy plant. Diseased plants set fewer pods and fewer peas per pod than healthy plants, and thus the yield is reduced. The symptom most significant to this study, however, is that the peas on diseased vines reach and pass the canning stage more quickly than those on healthy vines. In this way hard peas sometimes find their way into the pack.

The term "root rot" is used in this paper to denote a symptom complex. It describes most aptly the morbid condition of the pea plant when its below-ground parts are infected with pathogenic microorganisms. Several such organisms are known to occur in the soil on the canning crops farm of the New York State Agricultural Experiment Station at Geneva, N. Y., where most of the pea samples for these tests were obtained. The disease under investigation can not be ascribed with certainty to any single organism. The most important of the root-rotting fungi present are, *Pythium* spp., *Aphanomyces eueiches*, Drechs., *Fusarium marini pisi*, F. R. Jones and other species of *Fusarium*. *Ascochyta pinodella*, L. K. Jones, *Mycosphaerella pinodes* (Berk. and Blox.) Stone, *Rhizoctonia solani* Kuhn, and species of bacteria and nematodes are also found sometimes in the roots, epicotyls, or hypocotyls of the diseased plants.

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<sup>1</sup> Received for publication Dec. 8, 1931, issued June, 1932.



## MATERIALS AND METHODS

Pea samples for this study were obtained from five fields spotted with root rot. In each field two areas of 10 to 20 square yards each were selected, one in a spot showing the well-known symptoms of root rot and the other in an adjacent spot where nearly disease-free plants were growing. A sample weighing approximately 3 kg was removed between 7 and 8 a. m. from each of these areas at various intervals, as shown in the tables. An attempt was made to get the control samples from normal-looking plants free from discoloration, although it must be stated that, as a rule, a small amount of root infection could be detected all over the field. Since the control sample consisted of disease-free or nearly disease-free plants, it is hereafter called normal.

An effort was made to trace the development of comparable peas during the canning season. Since, obviously, the same individuals could not be tested on more than one day, it was necessary to try to get comparable sections from the diseased and normal pea populations. This was done by using large numbers of vines (3 kg) and by harvesting these from contiguous areas as the season advanced.

The whole plants were always pulled, being broken at the ground line, and brought to the laboratory, where the pods were removed and shelled by hand. All samples and all determinations on the fresh peas themselves were finished within one hour after harvesting.

As soon as the sample could be shelled, it was passed through a set of hand sieves which separated it into the usual sizes 1-6, the diameters of which are given by Sayre, Willaman, and Kertesz (10). The total weight of peas in grams and the weight in each size were then determined. Also the total weight of vines and pods was found. From each size, where possible, quadruplicate lots of 20 unbroken peas were taken for the crushing test. The load necessary to crush each of these four lots was then determined by using Green's (4) modification of the crushing tester proposed by Sayre, Willaman, and Kertesz (10).

From these experimental data the several factors as shown in the tables were calculated. The average crushing load for the four lots of any one size was reduced to kilograms per pea. The quality index, here called *QI*, was calculated by adding for all sieve sizes, except size 1, the products obtained by multiplying the average crushing load of each size by the percentage of peas in that size. Boswell's number (1) or maturity index, here called *MI*, was calculated as he suggested by adding the products obtained by multiplying the percentage of peas by weight in each size by the size number. The data in the column in the tables headed "Yield of fresh peas per 100 g of vines" were obtained from the respective weights of the peas and the vines in any one sample.

For the determination of the dry matter of the peas 25 g or 50 g samples were used. These samples were dried at 100° C for two days, after which several controls failed to show a further decrease in weight. These dried samples were used later for determining the ash, nitrogen, and crude fiber by the official methods.

## OBJECT OF THE INVESTIGATION

This paper reports a study made during the 1931 season of some of the internal chemical changes that took place in the ovules of pea plants affected with root rot. The investigation was designed primarily as a study of the aberrations in ripening and quality of canning peas that result from root rot. It is an established fact that pea root rot affects the production of canning and market garden peas, but so far as the writers are aware, this is the first attempt to evaluate the effect of root rot on the ripening and quality of peas. The significance of the maturity index of Boswell (1)<sup>2</sup> and of the quality index proposed by Sayre, Willaman, and Kertesz (10) was examined with reference to root-rot-affected peas.

## LITERATURE REVIEW

The literature of plant pathology includes few references to the internal chemical changes that occur in plants as a result of disease. The results obtained by Shutt (12) and, independently, by Snyder (13), when reduced to a dry-matter basis, show that the grain of rusted wheat plants is higher in ash, crude fiber, and protein than the grain of healthy plants. Shutt concluded that "the growth of the rust arrests development and induces premature ripening, which \* \* \* means a straw in which still remains the elaborated food, and a grain small, immature, rich in protein, and deficient in starch." Stoa (14) calculated his results on a basis of 13.5 per cent moisture, and concluded that the percentage of protein and ash decreases in the maturing grains of rusted wheat. Headden (5) agreed with Shutt and Snyder that the percentage of crude fiber is increased by wheat rust, but disagreed in that he found a decrease in percentage of protein.

Dungan (3) inoculated corn in alternate hills at planting time with various root-rotting fungi, and then analyzed the grain at harvest time. He found a decrease in specific gravity from 1.181 in the uninoculated plants to 1.154 in the inoculated. Furthermore, the inoculated plants were significantly different from the uninoculated in that they contained more nitrogen but less ether extract and total sugar. These differences were shown to be statistically significant.

The water relations of diseased plants are somewhat better known than the chemical ones. Weiss (15) found that the so-called water requirement of wheat plants infected with rust is higher than that of healthy plants. Kursanov (7) found a greater transpiration of wheat plants infected with *Ustilago tritici*, the ratio being 1.12. Linford (8) determined that peas affected with the wilt disease caused by *Fusarium orthoceras* var *pisi* have a higher percentage of dry matter than the healthy peas. Horsfall (6) showed that the stunting of clover plants affected with powdery mildew was probably due to the fact that the protoplasm of the diseased plants was functioning under drier conditions than that of healthy plants. Shapovalov and Jones (11), working with artificially inoculated plants, confirmed field data obtained by Rosa (9) showing that tomato plants affected with yellows were higher in dry matter than healthy plants.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p. 848

affected samples in yield in grams of fresh peas per 100 g of fresh vines, whereas in the normal samples the weight of fresh peas on this basis did increase

Two other samples of Alaska peas, one from a commercial field, were obtained. These showed similar differences between normal and root-rot-affected peas. Since both of these samples were taken only on one day each, they do not by themselves give a true picture of the growth and ripening of the peas. In all these samples the root-rot-affected peas always required a higher crushing load and had a higher dry-matter content than the corresponding sample of normal peas of the same size.

#### ADVANCER PEAS

The samples of Advancer peas came from a commercial field near Hall, N. Y., on June 30, July 3, and July 6. The field was harvested for Fancy peas on July 3. The results obtained are given in Table 2.

TABLE 2—Comparison of Advancer peas from normal and from root-rot-affected vines on different dates

Date of harvest	Normal						Root rot affected					
	Size No	Distri- bution of sizes	Crush- ing load	QI <sup>a</sup>	MI <sup>b</sup>	Dry matter as percent- age of—	Size No	Distri- bution of sizes	Crush- ing load	QI <sup>a</sup>	MI <sup>b</sup>	Dry matter as percent- age of—
						Peas Vines						Peas Vines
June 30.....	1	Per cent	Kg per pea				1	Per cent	Kg per pea			
	2	12 1	1 38	203 2	274 2	18 30	2	4 7	1 77	247 2	287 5	21 43
	3	17 2	2 45			24 09	3	25 2	2 74			34 97
	4	56 3	3 14				4	48 0	3 23			
	5	13 2	1 2				5	22 1	3 23			
July 3.....	1	4 5		300 4	380 1	17 42	1	5 7	2 41	317 1	378 0	22 80
	2	6 3	1 82			18 44	2	5 9	3 04			24 84
	3	18 5	2 87			20 80	3	26 6	3 27			25 24
	4	40 1	3 15			21 89	4	49 3	3 65			
	5	26 5	3 55				5	16 5	3 65			
	6	4 1	3 77				6	1 0				
July 6.....	1	1 3		409 9	455 9	18 80	1	1 2	2 92	400 7	392 5	31 30
	2	1 4	2 04			22 32	2	6 3	3 81			30 10
	3	4 8	3 67			26 72	3	25 0	4 37			30 42
	4	34 0	4 02				4	38 8	4 52			
	5	49 6	4 32				5	23 3				
	6	8 9	4 34				6	5 4				

<sup>a</sup> Quality index

<sup>b</sup> Maturity index

Root rot influenced the quality of the Advancer peas in the same way as it did that of Alaska peas. Exceedingly hot weather prevailed during the period of enlargement of these peas, and no rain fell during the two weeks previous to July 2. As a result of these conditions the peas moved toward the canning stage very rapidly, as is shown by the remarkable increase in the MI value for both normal and diseased peas.

Here, again, the MI value of the normal peas continued to increase after it had become practically stationary in the root-rot-affected peas. The samples of July 6 were far below Standard grade peas. In all sizes taken on June 30 and July 3, where the two factors were determined together, the crushing loads and the dry-matter contents of the diseased samples were higher than those of the normal samples. The results obtained on the larger sizes of the samples taken on July 6 are irregular because the crushing loads obtained at that time were very near the highest limit of the machine used.

## PRESENTATION OF RESULTS

## MATURITY AND QUALITY OF NORMAL AND ROOT-ROT-AFFECTED PEAS

## ALASKA PEAS

Samples were obtained from a 1.5-acre field of Alaska peas planted on April 21, 1931, on the canning crops farm of the State experiment station.<sup>3</sup> The root rot occurred on the edge of the field only, in a strip 4 to 5 feet wide. On account of dry weather the peas ripened quickly but gave a good yield of Fancy grade peas. Samples were taken from the normal and from the root-rot-affected areas on June 22, 24, and 26. On June 28 one further sample was taken from the normal peas. The results obtained from this series are presented in Table 1.

TABLE 1—Comparison of Alaska peas from normal and from root-rot-affected vines on different dates

Date of har- vest	Normal						Root rot affected								
	Size No	Distribution of sizes	Crushing load	QI <sup>a</sup>	MI <sup>b</sup>	Dry mat- ter as per- centage of—		Size No	Distribution of sizes	Crushing load	QI <sup>a</sup>	MI <sup>b</sup>	Dry mat- ter as per- centage of—		
						Peas	Vines						Peas	Vines	
															Yield of fresh peas per 100 g of vines
June 22	(169 9 230 1)	(1 01)	30 4	130 1				(1 25 3 2 46 0 3 28 7 1 12 2 2 30 5 3 44 3 4 13 0 1 7 8 2 35 6 3 43 0 4 13 6	(1 45 1 96 1 87 2 32 1 87 2 32 2 50 1 85 2 31 2 95 3 18	122 9	203 4		Gms		
June 24	(144 1 230 7 325 2)	(1 41 1 99)	93 5	181 1	19 55	7 36					192 3	258 1	24 60	17 64	
June 26	(136 7 173 2 321 7 48 8 114 7 217 9 330 2 429 7 57 5	(1 66 1 66 2 24 2 53 1 82 2 51 3 18 3 04	125 3	202 6	(17 40 20 67 23 74 24 37 20 12 24 10 25 69 32 05	21 48	14 05				252 3	262 4	(24 25 26 18 30 82 30 86)	33 12	18 68
June 28			224 5	297 4			20 90	23 49							

<sup>a</sup> Quality index<sup>b</sup> Maturity index

It is apparent that the peas increased in size very rapidly. On June 24 no peas of size 4 were found in the normal part of the field, but on June 26, 8.8 per cent and on June 28, 37 per cent of the peas harvested were size 4 or larger. While the normal peas hardened rather slowly, those affected with root rot ripened quickly. They attained a given size much earlier than the normal peas, but they did not enlarge much further. This fact is shown by the changes in the MI and QI values of these samples. In the case of the normal peas there is a steady increase in both values. In the peas from the affected area the MI value (representing enlargement) increased only from 258 to 262 after the second sample was taken. In spite of this fact the QI value increased considerably, showing the rapid hardening of the peas. There was no rainfall between June 22 and June 28. The dry-matter content of the normal plants and peas increased slowly, but that of the root-rot-affected plants and peas increased rapidly. During this interval of time there was no significant increase in the root-rot-

<sup>3</sup> For these samples and for the Perfection pea samples discussed later the writers are indebted to the vegetable crops division.

Figure 1 shows the relation between crushing load and sieve size of peas. Each point represents the average crushing load of any given size irrespective of age. The numbers beside each point represent the average dry-matter content. It is clear from these curves, as was already well known, that the diseased peas, size for size, are harder than the normal. It seems significant, however, that the difference in crushing load between normal and diseased peas for any one size is approximately the same as that for any other size, even though the dry-matter content varies tremendously.

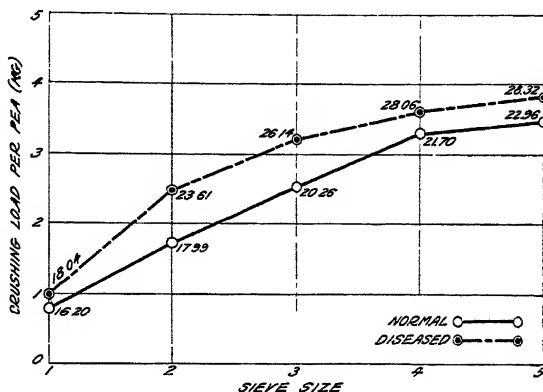


FIGURE 1.—The relation, in normal and diseased Perfection peas, between crushing load and sieve size of peas as averages for five harvest dates, showing that, size for size, the diseased peas are harder than the normal. Small figures beside the points show dry-matter content.

The increase in crushing load with age of diseased and normal peas is shown in Figure 2. The points on the curves represent average crushing loads for all pea sizes weighted according to the diameter of the size in millimeters. From Figure 2 it appears that the crushing load of the normal peas increased fairly regularly with age, but the crushing load of the root-rot-affected samples was lower on July 3 than on the

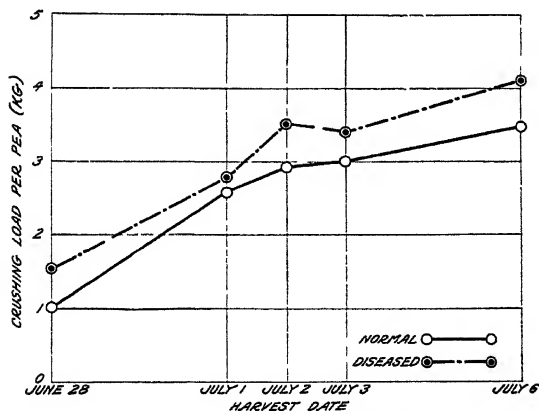


FIGURE 2.—Increase in crushing load with age, for normal and diseased Perfection peas harvested on different dates. The points on the curves represent averages for all pea sizes weighted according to the diameter of the size expressed in millimeters.

previous day. This can be explained only as the result of a heavy rain (0.99 inch) that fell on the evening of July 2. Sayre, Willaman, and Kertesz (10), working only with normal Perfection peas, found also that a rain of 0.56 inch did not reduce the load necessary to crush them. The lowered crushing load of the diseased peas after the rain would, therefore, appear significant.

The increase in dry-matter content with age of diseased and normal peas appears in Figure 3. The points on the curves represent average dry-matter content weighted, like the crushing load in Figure 2, according to the diameter of the size in millimeters. It should be noted that the drop in dry-matter content of the diseased peas after the rain of July 2 was much more pronounced than in

In view of the excessive dryness within the peas on the diseased vines, that contributed in part to their relative hardness, it seemed worth while to see if a part of this was not due to the withdrawal of water from the peas by the leaves. Accordingly the writers made use of the technic devised by Chandler (2) to demonstrate that leaves of fruit trees can extract water from the fleshy fruits. A dozen vines from each of the samples collected June 30 and July 3 were divided into two lots of six vines each, the pods being removed from six vines and left attached to six. The vines from which the pods were removed wilted approximately one hour sooner in each case than did those from which the pods were not removed, showing that leaves do have the capacity to withdraw water from the peas, thus aggravating a dry condition induced by a curtailed power of absorption through the diseased roots.

#### PERFECTION PEAS

The observations on Perfection peas are the most complete obtained in this study. The peas were planted on April 15 on the canning crops farm of the experiment station. About one-third of the field was very badly injured by root rot. Because of this disease the yield obtained when the crop was harvested for Fancy peas (July 2) was much lower than the average yields previously obtained on this farm. The peas were in the Fancy grade on July 2, in the Extra Standard grade on July 3, and were Standard on July 6. The results obtained are presented in Table 3. The data that appear in Figures 1 to 8 were taken from Table 3.

TABLE 3—Comparison of Perfection peas from normal and from root-rot-affected vines on different dates

Date of harvest	Normal						Root rot affected							
	Size No	Distribution of sizes (Crushing load per pea)	Q1 <sup>a</sup>	M1 <sup>b</sup>	Dry matter as percent- age of —		Yield of fresh peas per 100 g of vines	Size No	Distribution of sizes (Crushing load per pea)	Q1 <sup>a</sup>	M1 <sup>b</sup>	Dry matter as percent- age of —		Yield of fresh peas per 100 g of vines
					Peas	Vines						Peas	Vines	
	Per cent	Kg					Gms	Per cent	Kg					Gms
June 28.....	176 20 77 32 21 2 93 3 2 61 36 1 17 0		23 2	126 4	16 20 15 45	19 22	1 50 1 3 31 9 18 0 2 06 7 7 7			82 4	167 9	18 04 19 14 19 83	27 51 9 7	
July 1.....	32 21 3 68 3 23 2 32 27 9 2 88 3 9 3 06 6 1 0 1 1 7		199 3	294 3	18 91 19 78 21 39 21 78	22 98	1 22 2 3 37 5 22 2 1 78 26 2 2 69 6 3 16 1 3 25		243 0	301 7	20 89 22 07 23 35 23 63	27 0 19 9		
July 2.....	2 18 71 98 3 28 2 88 4 40 3 34 5 10 5 30 6 2 2 1 3 3		258 8	339 8	18 91 20 14 21 37 23 34	25 20	1 26 9 3 19 2 43 9 3 50 5 4 4 72 5 9 3 76 6 9 4		337 7	331 1	30 79 29 81 28 85	50 01 21 1		
July 3.....	2 4 51 92 3 28 92 97 4 41 0 28 5 21 83 49 6 5 5 1 7		305 0	375 0	18 68 19 80 20 71 22 11		1 3 2 3 26 5 4 45 4 5 23 3 6 1 2 1 3 1		346 6	391 6	28 70 27 20 28 21			
July 6.....	2 2 82 11 3 12 23 08 4 47 43 69 5 29 53 96 6 7 4 16		366 1	424 4	21 32 23 32 24 61		1 12 6 3 12 6 5 25 1 3 38 7 5 19 3 6 1 2		395 1	362 1	35 26 32 84 33 11			

<sup>a</sup> Quality index

<sup>b</sup> Maturity index

against each other irrespective of size or age of the samples. Reference to the curves in this figure shows, over the range in which they are comparable, that the crushing load for the peas of any particular percentage of dry matter averaged nearly 1 kg per pea lower for the root-rot-affected than for the healthy peas, thus confirming the conclusion drawn from Figure 4 that the structure or composition of the dry matter of diseased peas is less resistant to crushing than that of normal peas. Figure 5 also shows that the relation between crushing load and dry matter became much less distinct in the diseased samples when the dry matter was above 25 per cent. Unfortunately, no samples from normal peas were available to show what this relationship might have been when their dry matter was above 25 per cent.

The increase in the MI values in Table 3 is again very characteristic, showing that the increase in size of the diseased peas stopped before the normal peas attained their maximum size. The QI values of the root-rot-affected samples were always higher than those of the normal peas, on any given date. The true difference is not well expressed by these figures, however, since in the latter part of their growth the normal samples showed a higher percentage of the large sizes than did the root-rot-affected samples.

The yield of fresh peas per 100 g of fresh vines was almost the same in the root-rot-affected samples taken on July 1 as in those taken on July 2, showing that the peas did

not increase in weight at the expense of the vines as they did in the case of the normal samples, where this ratio increased materially. The dry-matter content of the normal vines increased between July 1 and July 2 at the usual rate, whereas, in the diseased vines, the percentage of dry matter nearly doubled, showing that the root-rot-affected vines were affected by the drought so that they dried out rapidly. Despite this decrease in the water content of the diseased plants, they did not wilt appreciably. As a result of a heavy rain (0.99 inch) that fell on the evening of July 2 the moisture deficiency of the diseased vines was somewhat relieved, as shown by the fact that the water content of the peas themselves actually increased.

On the sample of peas taken July 6, an additional test was made to determine whether pea leaves could withdraw water from the pods, thus contributing to the dryness of the peas within. This test confirmed the two reported for Advancer peas by showing that the vines without pods wilted much more quickly than those from which the pods had not been removed. Thus it is evident that the dryness of

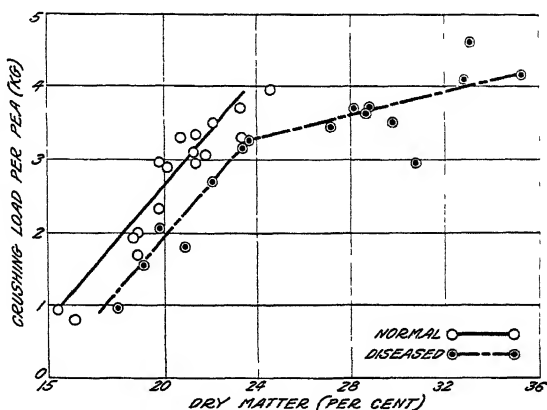


FIGURE 5.—The relation in normal and diseased Perfection peas between dry-matter content and crushing load, irrespective of sieve size or age. The diseased peas are softer than the normal on the basis of equal percentages of dry matter.

that of the normal peas. This drop in the percentage of dry matter for diseased peas is undoubtedly related to the decrease in the crushing load as shown in Figure 2 on the same day

In view of the work of Sayre, Willaman, and Kertesz (10), who

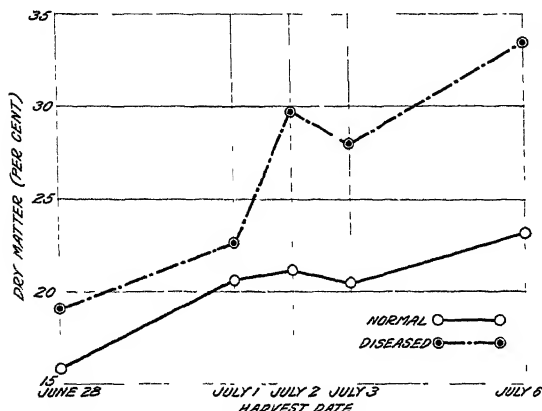


FIGURE 3—Increase in dry-matter content with age, for normal and diseased Perfection peas harvested on different dates. The points on the curves represent averages for all pea sizes weighted like the crushing load in Figure 2 according to the diameter of the size in millimeters. It seems significant that the drop in dry-matter content of the diseased peas after the rain of July 2 should have been so much more pronounced than that of the normal peas

showed that crushing load (they called it crushing test) bears a close relation to dry-matter content for normal peas, it seemed worth while to plot for diseased as well as normal peas the crushing loads from Figure 2

and the dry-matter contents from Figure 3 together, using a compound vertical axis scaled on the left according to dry matter in per cent and on the right according to crushing load per pea in kilograms. This has been done in Figure 4.

As was expected, the curves for dry-matter content and crushing load follow each other fairly regularly, as they should do if they bear a close relation to each other. For root-rot-affected peas, however, the dry-matter-content curve climbs relatively more rapidly with age than it should in relation to the crushing load.

With this particular combination of vertical scales, the crushing-load curve for the most part remains above the dry-matter curve for the normal samples. The crushing-load curve remains altogether below the dry-matter curve for the root-rot-affected samples, however. This indicates that the composition or structure of the dry matter of the diseased peas is less resistant to crushing than that of the normal peas.

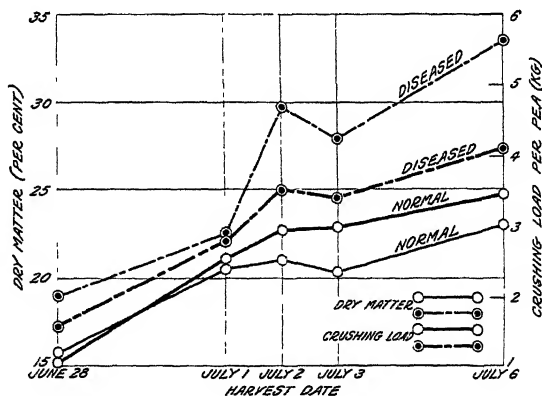


FIGURE 4—A combination graph in which the crushing load curves from Figure 2 are combined with the dry-matter curves from Figure 3 using a compound vertical axis scaled on the left according to dry matter in per cent and on the right according to crushing load per pea in kilograms. The dry-matter curve for the diseased peas seems to climb more rapidly than it should have in relation to crushing load, as shown by the normal peas

This indicates that the composition or structure of the dry matter of the diseased peas is less resistant to crushing than that of the normal peas.

In Figure 5 the direct relation between dry-matter content and crushing load for both diseased and normal peas are shown plotted

TABLE 5—Comparison of nitrogen content of normal and root-rot-affected pea samples harvested on different dates

ALASKA VARIETY					
Date of harvest	Size No	Nitrogen in normal peas		Nitrogen in peas with root rot	
		Wet basis	Dry basis	Wet basis	Dry basis
		Per cent	Per cent	Per cent	Per cent
June 26.....	1	0 91	5 24	0 98	4 06
	2	99	4 77	1 02	3 51
	3	1 08	4 54	1 09	3 54
	4	1 09	4 47	1 21	3 92
ADVANCER VARIETY					
June 26.....	4	1 02	4 87	1 07	4 29
	5	1 03	4 71	1 12	4 44
PERFECTION VARIETY					
July 1.....	2	0 965	5 10	0 972	4 65
	3	912	4 61	1 035	4 69
	4	996	4 66	1 078	4 61
	5	998	4 58	1 026	4 34
July 2.....	2	940	4 97	1 336	4 34
	3	988	4 91	1 339	4 49
	4	982	4 59	1 296	4 49
	5	1 111	4 76	-----	-----
July 3.....	2	1 020	5 46	-----	-----
	3	988	4 99	1 191	4 15
	4	1 030	4 98	1 195	4 40
	5	1 085	4 91	1 291	4 58
July 6.....	3	1 016	4 77	1 656	4 70
	4	1 101	4 72	1 451	4 42
	5	1 181	4 80	1 527	4 61

TABLE 6—Comparison of crude fiber content of normal and root-rot-affected Perfection pea samples harvested on different dates

Date of harvest	Size No	Crude fiber in normal peas		Crude fiber in peas with root rot	
		Wet basis	Dry basis	Wet basis	Dry basis
		Per cent	Per cent	Per cent	Per cent
July 1.....	2	1 36	7 26	1 54	7 38
	3	1 54	7 81	1 66	7 54
	4	1 72	8 03	1 94	7 79
	5	1 92	8 84	1 82	7 64
July 2.....	2	1 40	7 42	2 71	7 88
	3	1 70	8 42	2 41	8 09
	4	1 80	8 41	2 17	7 50
	5	1 95	8 36	-----	-----
July 3.....	2	1 39	7 44	-----	-----
	3	1 66	8 37	2 32	8 09
	4	2 00	9 35	2 27	8 35
	5	2 05	9 28	2 55	9 03
July 6.....	3	1 75	8 24	3 18	9 00
	4	1 94	8 34	3 15	9 59
	5	2 10	8 55	2 93	8 84

peas on root-rot-affected plants is partly due to the removal of water from them by the leaves, which are unable to obtain from the diseased roots all the water that they require for transpiration

#### CHEMICAL CHANGES IN NORMAL AND ROOT-ROT-AFFECTED PEAS

In order to obtain an insight into the changes taking place in some of the constituents of the normal and root-rot-affected peas, the percentages of ash, nitrogen, and crude fiber were determined in several of the samples. The results are presented in Tables 4, 5, and 6

TABLE 4—Comparison of ash content of normal and root-rot-affected pea samples harvested on different dates

ALASKA VARIETY					
Date of harvest	Size No	Ash from normal peas		Ash from peas with root rot	
		Wet basis	Dry basis	Wet basis	Dry basis
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
June 26.....	1	0 805	4 62	0 942	3 89
	2	859	4 16	940	3 22
	3	923	3 89	958	3 12
	4	950	3 90	1 153	3 74
ADVANCER VARIETY					
June 26.....	4	0 745	3 56	0 865	3 48
	5	755	3 45	926	3 67
PERFECTION VARIETY					
July 1.....	2	0 827	4 37	0 840	4 02
	3	884	4 47	843	3 82
	4	840	3 83	869	3 72
	5	888	4 08	940	3 97
July 2.....	2	892	4 72	1 28	4 17
	3	819	4 07	1 23	4 12
	4	807	3 78	1 03	3 57
	5	901	3 86		
July 3.....	2	853	4 57		
	3	844	4 26	989	3 45
	4	880	4 10	940	3 45
	5	925	4 19	948	3 36
July 6.....	3	886	4 16	1 28	3 64
	4	914	3 92	1 06	3 23
	5	972	3 95	1 12	3 34

The percentage of ash on a fresh-weight basis was slightly higher in the root-rot-affected samples than in the controls. On a dry-matter basis, however, the percentage of ash in the diseased samples was nearly always lower, except in the Alaska sample, where it was practically the same as in the normal peas. Nitrogen also in the root-rot-affected peas was lower on a dry-matter basis than in the normal peas. No significant differences were observed in the crude-fiber content of the dry matter in the samples of Perfection peas.

normal plants This condition was shown by Headden (5) to obtain when wheat is severely rusted This conclusion is supported also by the fact that the MI values showed that the diseased peas failed to enlarge materially during the same period It appears, then, that the peas began to dry out before they received their quota of filling material and were thus harder size for size than the normal peas, just as shown in Figure 1

The question arises, why, if the diseased peas are harder size for size than the normal peas, do the curves in Figure 5 show them to be softer? Since Figure 5 was drawn to show the relation between dry matter and crushing load, the explanation must hinge on some aspect of the dry matter An increase in percentage of dry matter in normal peas is associated with a definite increase in volume (Fig 6) In the case of diseased peas, on the other hand, an increase in the percentage of dry matter is not attended by as large an increase in volume

as in the normal peas The result is that for equal percentages of dry matter a normal pea is larger and therefore harder to crush than a diseased one For example, in Table 3 on July 1, a dry-matter content of 22.07 per cent was found in size 3 diseased peas that crushed at 2.69 kg per pea The nearest approach to 22 per cent dry matter in the normal peas on that date was 21.78 per cent, which occurred in size 5 peas that crushed,

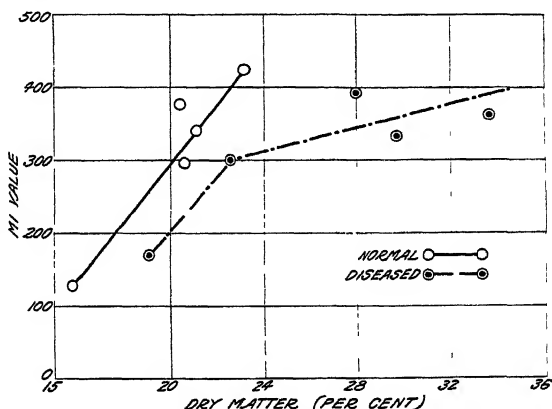


FIGURE 6—The relation, in normal and diseased Perfection peas, between MI (maturity index) and average dry-matter content, weighted like MI according to the sieve size number of the peas. The diseased peas are smaller than normal on the basis of equal percentages of dry matter, which in part explains why they are softer

not at 2.69, but at 3.06 kg per pea Assuming that the constitution of the dry matter was identical in the normal and diseased peas, then the fact that the normal peas of the same percentage of dry matter are larger than diseased peas would naturally help to explain why it is harder to crush them

Size alone, however, is not sufficient to account for the difference in the two curves It is illuminating in this connection to look in Table 3 not only for equal dry-matter content irrespective of size on the same day but also for equal dry-matter content of the same size irrespective of date, since neither date nor size is considered in Figure 5 The nearest approach to the 22 per cent dry-matter content for size 3 diseased peas on July 1 occurs in the normal peas on July 6 where peas containing 21.32 per cent dry matter crush, not at 2.69 kg, but at 3.08 kg per pea Since the sizes are known to be identical, it follows that the diseased peas containing 22 per cent of dry matter were softer than the normal peas containing 21.32 per cent Likewise size 3 diseased peas containing 19.83 per cent dry matter crushed at 2.06 kg per pea on June 28, a value matched in the

## DISCUSSION

Both the arrested growth and the diminished quality of root-rot-affected peas seem to be associated with the low water content of the diseased plants which are living under physiologically drier conditions than the normal plants in the same field. There seems to be little doubt that the stunting of the infected plants is due to the fact that the plants are unable to supply themselves with sufficient water to carry on properly the necessary metabolic functions. In the case of the stunting of clover affected with powdery mildew (Horsfall (6)), the water deficiency associated with the disease probably is induced by the excessive transpiration losses through the uncuticularized hyphae of the fungus covering the leaf. In the case of root-rot-affected peas, the deficiency seems to be due to a curtailment of the power of absorption by extensive root destruction.

The decrease in water content of the root-rot-affected peas militating toward a decrease in quality, as measured by the crushing test, may come about in two ways. (1) Unlike the normals, the diseased roots are unable to supply all the water lost by transpiration, especially under drought conditions such as prevailed during the 1931 harvest season. This of itself is sufficient to increase the rate of drying in the peas and thereby lower the quality. (2) The leaves, as already shown, have the capacity of offsetting a part of their own water losses by withdrawing from the pods water that they are unable to obtain through the diseased roots, thus contributing to the hardening of the peas within.

As previously stated, the crushing load and dry-matter content of the diseased Perfection peas actually diminished after the rain on the evening of July 2, whereas these values did not diminish materially for the normal peas (Figs 2 and 3). Apparently, the diseased peas absorbed enough water to replace a part of the deficiency, thus lowering the crushing load. The healthy peas, on the other hand, having no great deficiency, did not absorb enough water to lower the crushing load appreciably. The loss of water seems to be irreversible, or nearly so, in the normal samples, but not altogether irreversible in the case of the root-rot-affected samples.

In Figure 5, where the dry matter of diseased and normal peas is plotted against crushing load, irrespective of size or age of the samples, an apparently anomalous condition exists. The two factors appear to bear the same general relation to each other in both samples, as shown by the fact that the relation may be expressed by a line approximating a straight line in each case and that each line forms the same angle with the axes. Yet the curve for the diseased peas is lower for any particular percentage of dry matter by almost a kilogram per pea than for the healthy samples. A study of Figures 1 to 4 and the variation from time to time of the crushing loads of the various sizes furnished the justification for the plotting of Figure 5 without regard to size or age.

It seems significant that in both the Alaska and the Perfection samples the yield of fresh peas per 100 g of fresh vines did not increase materially beyond a certain stage in the diseased vines as it did in the normal. This indicates that the filling material either was not synthesized or did not move from the stems and leaves into the developing ovules of the root-rot-affected plants as freely as it did in the

are softer than the normal peas on the basis of equal percentages of dry matter

The maturity index as conceived by Boswell (1) does not fit the condition that obtains in a sample of root-rot-affected peas. Boswell's number has a significance, however, in comparing the size ratios of two samples of peas. It shows, for example, how the normal peas soon outstrip the diseased ones in enlargement. Boswell's number would rate normal peas as more nearly mature than the root-rot-affected ones of the same age that by reason of their greater dryness have more nearly reached the resting condition usually considered as maturity. It would also rate the diseased peas of the July 6 sample as less mature than those of the July 3 sample. The MI value is an admirable measure of size differences but not of maturity.

The quality index (QI) of Sayre, Willaman, and Kertesz (10) also fails in some measure to show the differences between the diseased and the healthy samples, because it, too, depends upon the size of the peas for its expression. This index could not be applied with success in a pea field where root rot is prevalent. Because of the actual decrease in average size for diseased Perfection peas this value is much lower on July 6 than it should have been.

#### SUMMARY

The effect of root rot on the physiology of peas, especially ripening and quality, was studied by following the changes in size distribution, load necessary to crush, dry matter, ash, nitrogen, and crude fiber in root-rot-affected peas as contrasted with normal peas.

According to the MI (maturity index) value, diseased peas enlarged more rapidly at first than healthy peas, but soon the rates of enlargement began to lessen, so that the diseased peas never reached the maximum size attained by normal peas.

After the growth of the diseased peas had begun to lessen their quality declined rapidly.

The curtailment of growth and the lowering of quality were both intimately associated with the lowered water content of root-rot-affected peas. This finding agrees with those of other investigators working with other plant diseases.

The load necessary to crush one pea was much higher, size for size, on the same harvest date in the diseased than in the normal samples. This is another way of saying that on the same harvest date, diseased peas were poorer in quality than normal peas. On the basis of an equal percentage of dry matter, however, the diseased peas were much softer than the normal peas. This was due to two facts. (1) They began to dry out before they were filled and thus they were smaller, hence their resistance to crushing was less, (2) the dry-matter stuff itself was softer than that in normal peas. Thus the relation between crushing load and dry matter was different in the root-rot-affected from that in the healthy peas.

No significant difference was found in the crude fiber content of normal and diseased Perfection peas.

The percentage of ash in the root-rot-affected peas on a dry-matter basis was always lower than that of the corresponding healthy peas. The percentage of nitrogen on a dry-weight basis was lower in practically every instance in diseased than in normal peas.

normal peas on July 1, where size 3 containing 19.78 per cent dry-matter crushed not at 2.06 but at 2.32 kg per pea. In explaining the difference on the basis of size, it was necessary to assume identical constitution of the dry matter. In explaining it on the basis of differences in structure or composition of the dry matter, assumptions are unnecessary, for the sizes are known to be identical.

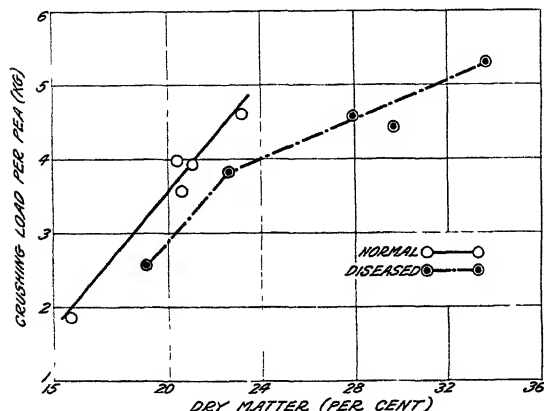


FIGURE 7.—The relation in normal and diseased Perfection peas harvested at different dates, between dry matter and crushing load, each being weighted according to the diameter of the peas expressed in millimeters. When the effect of size is thus eliminated the diseased peas are softer than the normal on the basis of equal percentages of dry matter.

tween dry matter and crushing load as those in Figure 5

Figure 7 represents an attempt to correct the crushing load and dry-matter content for size on the various harvest dates by weighting the

average for any particular day according to the diameter of the pea size in millimeters. In this way all determinations for each set of samples are reduced to one for each day. Here again, it may be seen that the diseased peas are softer, dry matter for dry matter, than the normal peas.

Figure 8 represents an attempt to correct the crushing load and dry-matter content for age by averaging the crushing loads for any particular size for

all dates. The small figures adjacent to the points show pea sizes. In plotting this figure the same calculations were used as for plotting Figure 1, except that dry matter instead of size appears along the horizontal axis. Figure 8 shows again that the diseased peas

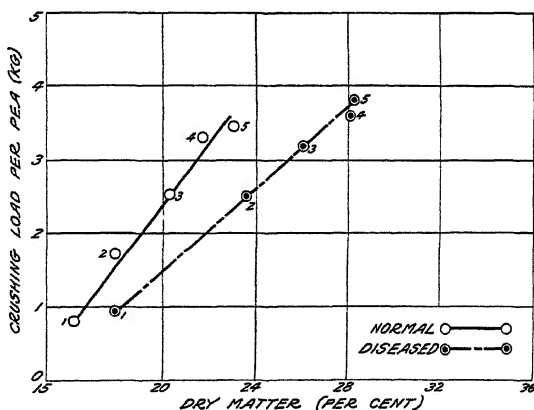


FIGURE 8.—The relation, in normal and diseased Perfection peas, between dry matter and crushing load as averages for all harvest dates. When the effect of age is thus eliminated the diseased peas are softer than the normal on the basis of equal percentages of dry matter. The small figures beside the points represent sieve size number.

# THE DISTRIBUTION OF VITAMIN B COMPLEX AND ITS COMPONENTS IN THE PEANUT<sup>1</sup>

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## INTRODUCTION

The original object of the experimentation reported in this paper was to determine the distribution of vitamin B in the various parts of the peanut kernel. At the time that the work was begun (1926) vitamin B, or water-soluble B, was quite generally considered to be a single substance, although several investigators had suggested its multiple nature. Within the next two years, however, definite proof that it contained at least two nutritive essentials had been published, and methods for their detection had been developed (8)<sup>2</sup>. Consequently the scope of this investigation was enlarged to include a study of the relative quantities of the antineuritic vitamin B (B<sub>1</sub> or F) and of the pellagra-preventing vitamin G (B<sub>2</sub>) present in whole raw-peanut kernels.

## HISTORICAL REVIEW

Ellis and McLeod (3, p 343) state that Vedder and Clark found, in 1912, that 10 g of peanuts per day protect fowls on a polished-rice diet against polyneuritis for at least 60 days. These authors also state (3, p 215) that in 1918 Grieg recommended groundnut (peanut) meal biscuit as emergency rations for the Indian troops.

In 1918 Daniels and Loughlin (2) published evidence to show that a ration containing 75 per cent of roasted Spanish peanuts contained sufficient water-soluble B for normal growth and reproduction of rats. They also found that 56 per cent of peanut meal in the ration furnished enough of this factor for their experimental animals.

Recently Plummer, Raymond, and Lowndes (7) have observed that pigeons suffered from polyneuritis when peanuts constituted 10 per cent of their ration. Twenty per cent of peanuts was enough for maintenance, and with 40 per cent rearing of young was possible. They conclude that peanuts have a relative vitamin B value of 20 when yeast is rated at 100. They say (7, p 546) that this

must at present be considered as the vitamin B<sub>1</sub>+vitamin B<sub>2</sub> value, though the symptom of polyneuritis has been taken as far as possible as the criterion of the amount of vitamin in the foods.

## DISTRIBUTION OF VITAMIN B COMPLEX IN THE PEANUT KERNEL

### PEANUT PRODUCTS USED

Raw unshelled peanuts, together with blanched splits, hearts, and red skins from extra-large selected Virginia Runner peanuts were obtained from one of the large mills.

<sup>1</sup> Received for publication Nov 18, 1931, issued June, 1932. Read before the biological section of the American Chemical Society at the Atlanta, Ga., meeting, Apr 8, 1930.

<sup>2</sup> Reference is made by number (italic) to Literature Cited, p 859.

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vitamin B complex free basal ration Each composite curve is derived from the behavior of from two to six, usually three, animals in a group. All experimental rats on a given ration reacted in a rather uniform manner The details for each individual are omitted in order to conserve space

The average daily feed intake of the rats on these rations shows that below a critical range in the percentage of peanut products in the ration the rats do not ingest sufficient vitamin B to affect their appetite to any great extent Within this range, however, an increase in the level of peanut product results in a much larger feed intake,

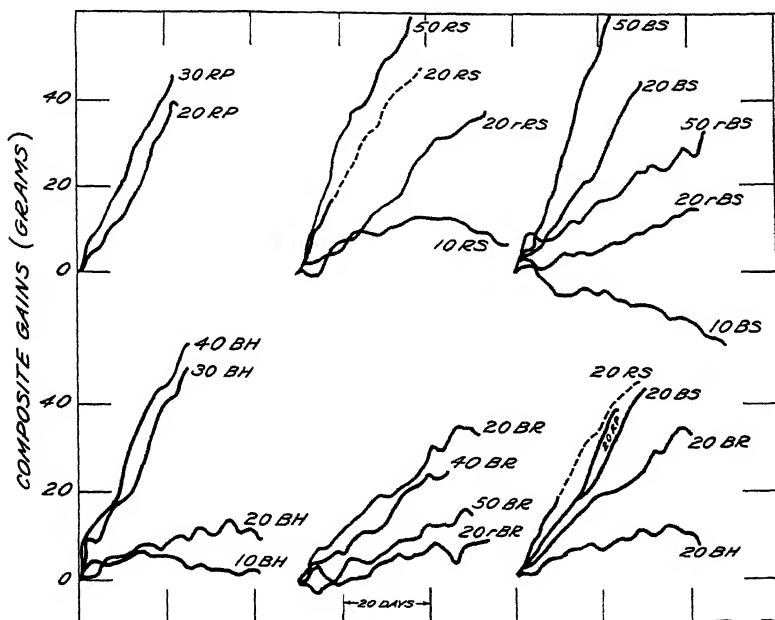


FIGURE 1.—Composite gains of rats when receiving peanut products incorporated in the ration as the sole source of vitamin B complex. The figures in the curve designations refer to the percentage of the peanut product in the ration, and the letters have the following meaning: RP, raw shelled peanuts, RS, raw splits, BS, blanched splits, BH, blanched hearts, and BR, blanched red skins. The letter r shows that the test was conducted on a product which was approximately 1 year old and had become rancid. The composition of the rations is given in the text.

consequently the daily ingestion of the peanut product increases proportionately much more rapidly than the percentage in the ration.

In order to control the daily intake of the peanut product another series of experiments was run in which definite amounts of the fractions were fed daily to individual rats

#### PEANUT PRODUCTS FED SEPARATELY

The same technic as that outlined above, when the peanuts were incorporated in the ration, was followed, except that the rats were separated at the end of the depletion period and kept in individual cages. They were then given daily weighed quantities of the peanut products, together with the basal ration and distilled water ad libitum.

The process of commercial blanching consists essentially in heating the graded kernels in oil at about 300° F until the seed coats, or red skins, are loosened from the underlying tissues. The heating is not sufficient to develop a brown color in the cotyledons. The red skins and the hearts (plumules and hypocotyls) are removed mechanically from the splits or fleshy edible cotyledons.

Since each of these commercial products contained a small amount of the other parts of the nut, before being used they were further separated by hand.

Some of the shelled raw kernels were spread in thin layers in a warm room until the seed coats had dried sufficiently to be rubbed off easily. These nuts were then divided by hand into raw splits, hearts, and red skins. It was found that these kernels were composed of about 95 per cent splits, 2.3 per cent hearts, and 2.7 per cent red skins.

#### EXPERIMENTAL WORK

##### PEANUT PRODUCTS INCORPORATED IN THE RATION

Rats from the experiment-station stock colony, when 22 to 28 days old and weighing 35 to 50 g, were placed in cages having raised screen floors and were given the basal vitamin B free ration (Table 1) and distilled water ad libitum for 10 to 20 days, until they had ceased growing. The lot was then given a ration which contained a definite amount of one of the peanut products. One lot of rats which received 20 per cent of shelled raw peanuts was given the ration without having had the preliminary depletion period. Records were kept of the approximate daily feed intake of each lot.

TABLE 1 —Composition of basal rations 195 and 196 and of supplementary ration 197 used in work on vitamin B complex

Ingredient	Composition of ration No —			Ingredient	Composition of ration No —		
	195	196	197		195	196	197
	Per cent	Per cent	Per cent		Per cent	Per cent	Per cent
Purified casein <sup>a</sup> .....	18.0	9	—	Osborne and Mendel's salt mixture.....	4.0	4	16
Meat residue <sup>b</sup> .....	—	12	48	Agar-agar.....	2.0	2	8
Purified starch.....	61.0	—	—	Hydrogenated vegetable fat.....	15.0	9	—
Washed starch.....	—	56	—	Cod-liver oil.....	60.15	6	28

<sup>a</sup> A nearly colorless powder free from vitamins A, B, and G, prepared according to the method given in N. C. Agr. Expt. Sta. Tech. Bul. 39 (4, p. 122).

<sup>b</sup> From a meat juice company.

<sup>c</sup> 0.15 g per rat per day.

The energy value of all of the experimental rations was approximately equal. These rations contained meat residue, 12 per cent; Osborne and Mendel salt mixture, 4 per cent; agar-agar, 2 per cent; and cod-liver oil, 4 per cent. The remainder consisted of the desired amount of peanut product, enough vegetable fat to make a total fat content of 25 per cent, and starch to make 100 per cent. The ration containing 50 per cent of blanched splits was an exception in that it contained 29.7 per cent of fat without the addition of a vegetable fat.

Figure 1 shows graphically the composite growth response obtained when these rations were fed to rats which had ceased growing on the

what poorer growth even though the average daily intake of this peanut product was 2.3 g per rat. The red skins seemed to be very unpalatable. The failure of the rats to respond to the larger amounts of red skins in the ration is possibly due to the deleterious effect of the tannins or other astringents present. Cajon (1) has shown that the presence of tannins in pecan diets is a limiting factor for the growth of rats when pecan nuts furnished the sole source of protein in the rations.

The larger part of the work with peanut products incorporated in the ration was completed in the early part of the summer of 1927. The following spring, after the peanut products had become somewhat rancid, the work with the rations containing 20 per cent of blanched red skins, 20 per cent of blanched splits, and 20 per cent of raw splits was repeated. In each case the growth response of the rats was dis-

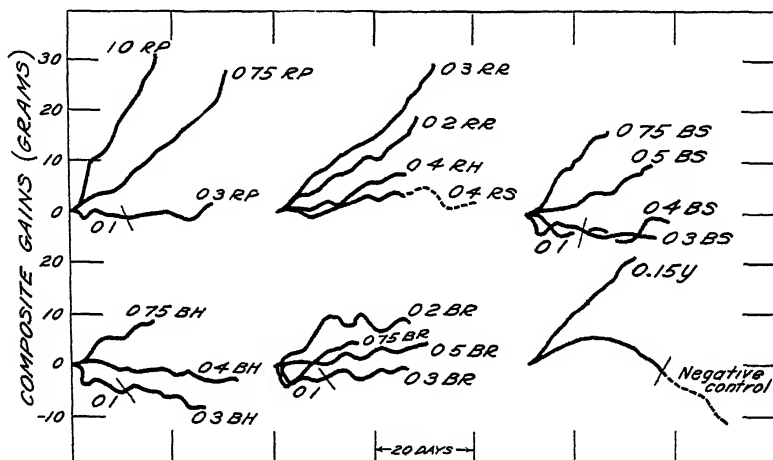


FIGURE 2—Composite gains of rats when receiving daily weighed quantities of peanut products as the sole source of the vitamin B complex. In the curve designations the figures refer to grams of the peanut fraction fed daily, and the letters have the following meanings: RP, shelled raw peanuts, RS, raw splits, RH, raw hearts, RR, raw red skins, BS, blanched splits, BH, blanched hearts, BR, blanched red skins, and Y, dried brewers' yeast. The composition of the basal rations (Nos. 195 and 196) is given in Table 1 and discussed in the text.

tinctly inferior to that obtained the preceding year (fig. 1), although the average daily feed intake was approximately the same. This indicates that the vitamin B complex was partially destroyed by storage at room temperature and that the rancidity did not affect the palatability of the rations to any marked extent.

The data (figs. 1 and 2) show that while the vitamin B complex is distributed throughout all parts of the peanut kernel, the raw red skins contain distinctly more of these factors than do the other parts. The raw hearts and raw splits apparently are about equally rich in these essentials.

Each of the blanched products contains somewhat less vitamin B complex than the corresponding raw product. This is particularly evident in the red skins but less so in the hearts and splits. The heating during the blanching process apparently destroys a large part of the thermolabile antineuritic vitamin in the thin exposed red skins but does not affect that in the body of the kernel to so great an extent.

Basal ration 195, used in the earlier part of this work, is very similar to those used for this type of work. It was found by experiment to be free from the vitamin B complex. Later, after careful preliminary experiments, basal ration 196 was substituted in order to reduce the time and expense incurred in the elaborate purification of the casein and starch (Table 1).

Osborne, Wakeman, and Ferry (6) and Sherman and MacArthur (10) have shown that commercial cornstarch does not contain the vitamin B complex. This observation has been confirmed in respect to the particular brand of starch used here, but as a precaution the raw starch was suspended in water and strained through a double layer of cheesecloth. After the starch had settled overnight, the water was decanted, and the starch was spread out in shallow pans and dried at room temperature. This material is designated as washed starch.

The meat residue used in ration 196 is a dry granular by-product from the commercial manufacture of beef extract. It contains approximately 80 per cent protein ( $N \times 6.25$ ) and 10 per cent fat, and is apparently free from the antineuritic vitamin and possibly vitamin G.

In the greater part of the work rations 195 and 196 were relied upon to furnish all needed nutritive essentials for growth except the vitamin B complex. It was feared that at times the rats might not eat enough of ration 196 to supply their minimum needs, so 0.25 g of ration 197 was fed daily with the peanut product (Table 1), the basal ration being withheld until all of the supplements were eaten. This procedure insured the consumption of at least 70 mg of cod-liver oil and 40 mg of the salt mixture each day. This method of feeding was discontinued after a short time, since there was no apparent benefit from it.

Before being fed, all peanut products, except red skins, were ground in a meat chopper, care being taken to avoid crushing them to the consistency of a paste. The red skins were finely ground in a Wiley mill.

Composite gains of the animals obtained when definite quantities of the raw and blanched fractions were fed daily are shown graphically in Figure 2. For the sake of brevity the details for each rat are not given. The curves are each the composite of three to five individually fed rats which reacted rather uniformly.

It was necessary to mix the unpalatable red skins with as much of the basal ration as would be consumed in a day in order to get the rats to eat them. The other fractions of the peanut kernel were eaten greedily.

#### DISCUSSION OF RESULTS

Figure 1 shows that neither 10 per cent of raw splits, blanched splits, nor blanched hearts stimulated growth in the rats. When, however, the amount of raw or blanched splits was doubled (that is, increased to 20 per cent) the rats responded promptly and made good growth. Because of their improved appetite these rats ate approximately four times as much of the splits as those receiving 10 per cent in their ration.

A ration containing 20 per cent of blanched hearts was only slightly superior to the one containing 10 per cent; but when the level was increased to 30 per cent, good growth resulted.

The rats receiving 20 per cent of blanched red skins made moderate growth. Increasing the red skins to 50 per cent resulted in some-

## EXPERIMENTAL RESULTS

## CONTROLS

In all, 68 rats were used as controls and in determining the suitability of the autoclaved yeast and of the extract of rice polish as

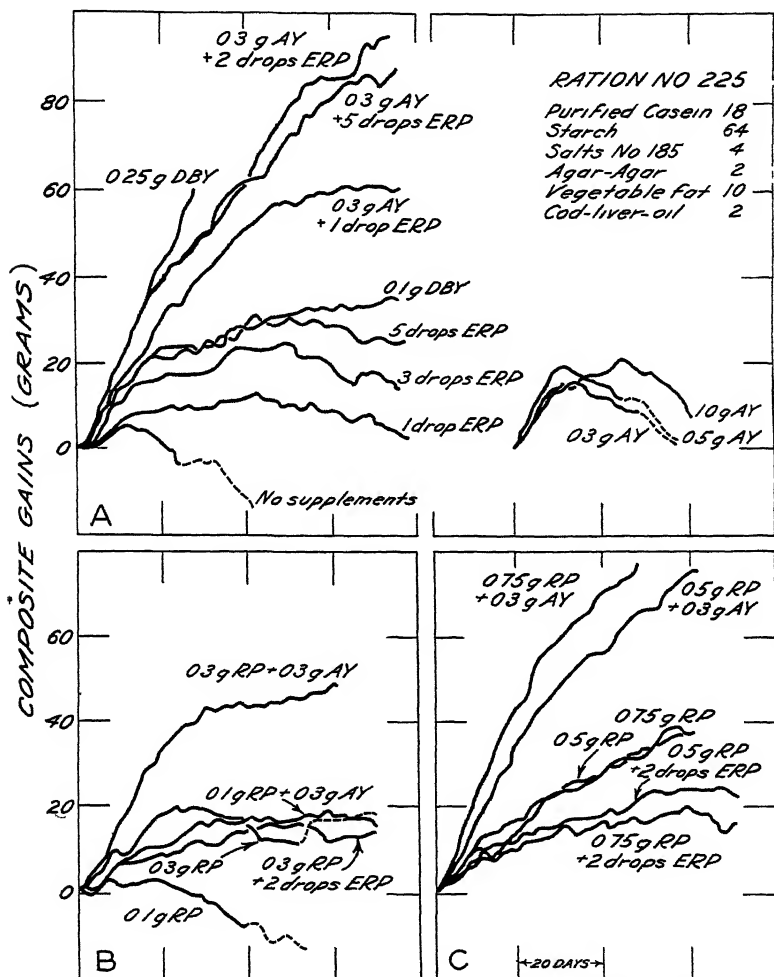


FIGURE 4—Composite gains of control rats (A) receiving the polyneuritic ration No 225, without supplements, or with daily definite quantities of autoclaved yeast (AY), extract of rice polish (ERP), or dried brewers' yeast (DBY), B and C, composite gains of experimental animals receiving daily ration No 225 with definite quantities of raw shelled peanuts (RP) with and without 0.3 g autoclaved yeast and 2 drops of extract of rice polish. The dotted portion of the curves indicates that one or more of the rats had died.

sources of vitamins G and B, respectively, and also the quantities that it is necessary to use for this purpose. The principal results are given in Table 3, and curves of composite gains are shown in Figures 3 and 4.

The hearts, being small and partially exposed at the end of the nut, are affected slightly more than the splits, but much less than are the red skins

# RELATIVE QUANTITIES OF THE ANTINEURITIC AND THE PELLAGRA-PREVENTING VITAMINS IN RAW SHELLED PEANUTS

## METHOD

The general method of Hunt and Krauss (5) was adopted as being suitable for this work, except that dried brewers' yeast which had been autoclaved at 20 pounds pressure for 4 hours was used as the source of the antipellagra vitamin (Sherman and Axtmayer (9)) These investigators used the two basal rations shown in Table 2. One of these, the polyneuritic ration, 225, is devoid of both components of the vitamin B complex. The other, 223, contains the antineuritic fraction and small amounts of vitamin G. During the

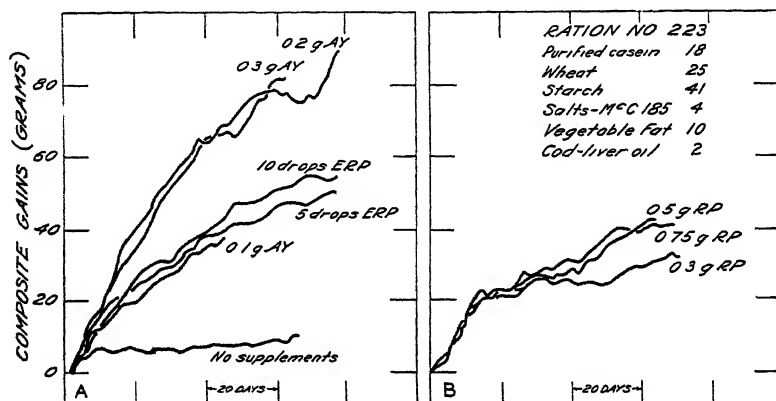


FIGURE 3—Composite gains of rats receiving the pellagra-producing ration No. 223 without supplements, with definite quantities of autoclaved yeast (AY), or extract of rice polish (ERP) or with raw shelled peanuts (RP) fed daily. A, Controls, B, animals fed peanuts

course of this work it was found that the polyneuritic ration supplemented with an extract of rice polish was more satisfactory than the pellagra-producing ration for detecting the presence of vitamin G, because of the small but appreciable amount of this vitamin furnished by the wheat in the latter ration. The extract of rice polish was prepared in this laboratory according to the method described by Wells (11) for the preparation of tikitiki.<sup>3</sup>

TABLE 2—Composition of basal rations used in work on the antineuritic and pellagra-preventive vitamins

Ingredient	Pellagra-producing ration No 223	Polyneuritic ration No 225	Ingredient	Pellagra-producing ration No 223	Polyneuritic ration No 225
	Per cent	Per cent		Per cent	Per cent
Purified casein.....	18	18	Agar-agar.....	—	2
Wheat.....	25	—	Hydrogenated vegetable fat	10	10
Washed starch.....	41	64	Cod-liver oil.....	2	2
McCormick's salts mixture 185.	4	4			

<sup>3</sup> Tikitiki is a concentrated extract of rice polish which is prepared and distributed by the Philippine Public Health Service for the prevention of beriberi

These control tests of the rations and of technic show that although young rats usually die within 30 days when given the polyneuritic ration only, a daily supplement of 1 drop of the extract of rice polish enables them to survive at least 80 days. However, they do not grow, and incipient symptoms of pellagra<sup>4</sup> develop within 50 to 70 days. When the dosage of extract of rice polish is increased to 5 or more drops slight growth results, indicating that this supplement may contain very small amounts of vitamin G, but that 2 drops daily does not furnish a sufficient quantity to be of any consequence.

The results also show that the autoclaved yeast contains only minute quantities of vitamin B. When the polyneuritic basal ration is supplemented with 2 drops of extract of rice polish and 0.3 g autoclaved yeast daily the rats are able to grow at the rate of approximately 1 g per day and maintain their health throughout the experimental period.

#### RATS FED PEANUTS

When tests for the presence of the antineuritic and antipellagra vitamins were made on rats fed shelled raw peanuts, the results shown in Table 4 and Figures 3 and 4 were obtained. Extra-large peanuts of the Virginia Runner variety were used. These were obtained from a different mill from the one which supplied the blanched-peanut products used in the earlier work on the vitamin B complex.

TABLE 4—*Effect of shelled raw peanuts on the growth of young rats receiving the polyneuritic basal ration alone, and supplemented either with autoclaved yeast or extract of rice polish, and their effect on the growth of rats receiving the pellagra-producing basal ration*

POLYNEURITIC BASAL RATION NO. 225 WITH NO ADDITIONAL SUPPLEMENTS

Rat No. and sex	Age at start		Weight at start		Quantity of raw peanuts fed daily	Maximum weight attained	Time after start when maximum weight was attained	Duration of test period	Weight at end of test period	Average quantity of basal ration eaten daily	Average gain or loss in weight per day	Pellagra symptoms	Remarks
	Days	Grams	Grams	Grams			Days	Days	Grams	Grams	Grams		
2401 ♀	26	48	0 10	52	30	8	38	34	34	34	0.37	—	Died Do Do
2402 ♂	24	50		58		6	53	34	39	39	.30	—	
2403 ♀	26	34		36		2	64	24	28	—	.15	—	
2098 ♀	23	44	30	64	50	54	83	60	40	40	.19	—	—
2145 ♀	24	50		64		28	70	58	33	33	.11	—	
2146 ♀	24	46		72		53	70	64	33	33	.26	—	
2039 ♂	21	48	50	92	104	60	60	92	43	43	.73	—	—
2040 ♂	22	46		68		44	60	68	37	37	.37	—	
2099 ♀	23	52		104		80	83	98	47	47	.55	—	
2041 ♀	22	46	75	68	86	60	60	68	40	40	.37	—	—
2042 ♂	25	42		86		56	60	56	45	45	.23	—	
2100 ♂	23	50		118		83	83	118	48	48	.82	+	

<sup>4</sup> The term "pellagra" is used to denote vitamin G avitaminosis. It does not necessarily imply a condition identical with pellagra in man. The extreme conditions described in the literature have not been encountered in this work. The most frequent symptoms have been emaciation and ophthalmia. Sores at the corners of the mouth and considerable loss of hair have been fairly frequent. A mild dermatitis has developed occasionally, and diarrhea has been noted in a few instances.

TABLE 3—Effect of autoclaved yeast and extract of rice polish as supplements to the pellagra-producing and the polyneuritic basal rations when fed to controls

PELLAGRA-PRODUCING RATION NO 223 WITH NO SUPPLEMENT											
Rat No and sex	Age at start	Weight at start	Quantity of supplement fed daily	Maximum weight attained	Time after start when maximum weight was attained	Duration of test period	Weight at end of test period	Average quantity of basal ration eaten daily	Average gain or loss in weight per day	Pellagra symptoms	Remarks
	Days	Grams		Grams	Days	Days	Grams	Grams	Grams		
1973 ♂	24	46	-----	64	64	64	64	-----	0 28	—	
1975 ♀	24	46	-----	54	12	64	44	-----	— 03	—	
1981 ♂	22	48	-----	58	9	46	46	-----	— 04	++	
1982 ♀	22	44	-----	60	42	46	60	-----	35	++	
1983 ♀	22	44	-----	74	46	46	74	-----	65	++	
PELLAGRA-PRODUCING RATION NO 223 WITH AUTOCLAVED YEAST											
2090 ♂	23	50	0.2 g -----	164	80	84	158	7 5	1 28	—	
2091 ♂	23	48		122	80	84	118	6 7	83	—	
2179 ♀	22	42	0.5 g -----	130	61	63	130	7 8	1 39	—	
2024 ♀	24	44		118	48	48	118	7 7	1 54	—	
PELLAGRA-PRODUCING RATION NO 223 WITH EXTRACT OF RICE POLISH											
2212 ♀	26	50	10 drops-----	108	78	83	108	7 0	70	—	
2224 ♀	26	44		94	66	76	90	6 5	61	—	
2248 ♂	26	40		102	69	76	100	6 1	79	—	
POLYNEURITIC RATION NO 225 WITH NO SUPPLEMENT											
2006 ♂	22	44	-----	48	10	26	34	-----	— 38	—	Died, spasms Experiment dis-continued
2007 ♂	22	42		48	7	26	34	-----	— 31	—	
2008 ♀	23	42	-----	42	0	26	28	-----	— 54	—	Died, spasms Experiment dis-continued
2009 ♀	23	38		40	12	26	28	-----	— 38	—	
2486 ♀	28	48	-----	52	9	47	32	3 8	— 34	—	Died, spasms
POLYNEURITIC RATION NO 225 WITH AUTOCLAVED YEAST											
2025 ♂	24	48	0.3 g -----	62	10	32	38	3 6	— 31	—	Died, spasms Experiment dis-continued, spastic
2071 ♂	22	40		52	15	45	36	2 9	— 09	—	
2072 ♂	22	42	1 g -----	56	15	43	36	3 3	— 14	—	Died, spasms Died
2028 ♂	24	40		48	10	45	40	2 3	00	—	
2084 ♂	23	42		76	24	43	44	3 4	05	—	Do Experiment dis-continued
2149 ♀	25	46		70	36	69	70	2 5	35	—	
POLYNEURITIC RATION NO 225 WITH EXTRACT OF RICE POLISH											
2214 ♀	26	48	1 drop -----	62	50	83	54	4 0	07	+	
2217 ♂	26	44		58	41	77	48	4 3	05	+	
2250 ♀	23	42	5 drops -----	56	34	76	40	4 3	— 03	+	
2216 ♀	26	50		78	27	83	68	4 8	22	+	
2219 ♀	26	44		74	41	77	64	4 9	26	+	
2232 ♂	22	40		82	45	76	80	5 2	53	+	
POLYNEURITIC RATION NO 225 WITH 0.3 G AUTOCLAVED YEAST AND 2 DROPS EXTRACT OF RICE POLISH DAILY											
2525 ♂	26	42	-----	154	90	90	154	5 6	1 24	—	
2586 ♀	25	56	-----	150	69	96	150	5 4	98	—	
POLYNEURITIC RATION NO 225 WITH DRIED BREWERS' YEAST											
2031 ♂	24	52	0.25 g -----	110	30	33	108	6 0	1 69	—	
2032 ♂	24	52		108	33	33	108	6 6	1 69	—	
2033 ♂	24	44		110	33	33	110	6 1	2 00	—	

than they did when the pellagra-producing basal ration containing 25 per cent of whole wheat was used. This indicates that the wheat in ration 223 (Table 2) contained enough vitamin G to interfere with the detection of the small amount of this factor in the peanuts.

It is recognized that vitamin B complex probably contains more than the two fractions (B and G) discussed here and that more definite knowledge concerning these other factors may necessitate a modification in the methods or a reinterpretation of the results. The conclusions expressed here are justified, however, unless it shall be proven that a serious error is introduced by the absence of one or more of these little-known substances from the peanuts or the basal ration or by their presence in the autoclaved yeast or in the extract of rice polish.

### SUMMARY

Commercially blanched peanut splits (cotyledons), hearts (plumules and hypocotyls), and red skins (seed coats), and the corresponding raw products from selected Virginia Runner peanuts were tested for the presence of vitamin B complex by a method which does not differentiate between the components, but in general favors the detection of the antineuritic fraction.

The raw red skins were found to contain the highest concentration of vitamin B complex, but there are appreciable quantities present in the hearts and the splits.

The process of commercial blanching (heating in oil for a short time at approximately 300° F) destroys a large part of the vitamin present in the outer exposed red skins but does not have so marked an effect on that in the hearts and splits.

When shelled raw kernels were tested for the presence of the components of vitamin B complex it was found that they contain relatively much larger amounts of the antineuritic fraction than of the pellagra-preventing vitamin G.

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TABLE 4—Effect of shelled raw peanuts on the growth of young rats receiving the polyneuritic basal ration alone, and supplemented either with autoclaved yeast or extract of rice polish, and their effect on the growth of rats receiving the pellagra-producing basal ration—Continued

POLYNEURITIC BASAL RATION NO 225 WITH 0.30 GRAM AUTOCLAVED YEAST PER RAT PER DAY

Rat No and sex	Age at start	Weight at start	Quantity of raw peanuts fed daily	Maximum weight attained	Time after start when maximum weight was attained	Duration of test period	Weight at end of test period	Average quantity of basal ration eaten daily	Average gain or loss in weight per day	Pellagra symptoms	Remarks
	Days	Grams	Grams	Grams	Days	Days	Grams	Grams	Grams		Died
2404 ♂	26	44	0 10	64	17	52	38	3 8	-0 12	—	
2405 ♂	24	46		68	27	86	64	3 9	21	—	
2406 ♂	26	40		64	22	87	66	3 6	23	—	
2184 ♀	22	42		56	47	60	73	4 3	60	—	
2190 ♀	23	44	30	110	60	62	108	4 9	97	—	
2182 ♀	22	38		90	56	61	90	4 3	76	—	
2185 ♀	22	38		112	49	60	112	5 4	1 23	—	
2185 ♀	22	46		124	60	62	122	4 9	1 22	—	
2191 ♀	23	44	50	118	59	61	116	5 6	1 18	—	
2183 ♀	22	34		128	61	63	126	5 4	1 46	—	
2186 ♀	22	46		146	60	62	142	5 9	1 55	—	
2192 ♀	23	36		120	56	61	116	5 6	1 31	—	

POLYNEURITIC BASAL RATION NO 225 WITH 2 DROPS OF AN EXTRACT OF RICE POLISH \* PER RAT A DAY

2410 ♂	24	46	0 30	82	69	78	74	4 4	0 36	—	
2411 ♂	24	40		52	24	78	44	3 7	05	—	
2412 ♀	24	48		56	6	78	44	3 9	-05	—	
2407 ♀	24	44		70	83	87	80	4 8	41	—	
2408 ♂	26	44	50	60	45	87	56	3 8	14	—	
2409 ♀	26	34		64	76	87	62	3 9	32	—	
2413 ♂	24	48		66	39	78	56	3 9	10	—	
2414 ♀	24	42		66	74	78	62	4 7	26	—	

PELLAGRA-PRODUCING BASAL RATION NO 223 WITH NO ADDITIONAL SUPPLEMENTS

2101 ♀	24	48	0 30	108	80	84	108	6 5	0 72	—	
2136 ♂	24	48		80	14	70	72	4 0	34	—	
2137 ♀	24	36		78	32	70	72	4 0	51	—	
2102 ♀	24	52		124	80	84	122	7 2	83	—	
2138 ♀	24	44	50	74	60	70	72	3 2	40	—	
2139 ♀	24	48		86	63	70	84	4 4	51	—	
2103 ♀	24	52		110	77	84	110	5 5	69	—	
2140 ♂	24	46		96	63	70	90	4 7	63	—	
2141 ♀	24	42	75	74	26	70	68	3 6	37	—	

\* One drop of extract of rice polish weighed on an average of 64 mg

#### DISCUSSION OF RESULTS

The results presented in Table 4 and Figure 4 show that the anti-neuritic vitamin contained in an extract of rice polish does not adequately supplement raw peanuts in inducing growth in young rats receiving a basal ration which is free from vitamin B complex. When, however, autoclaved yeast is substituted for the extract of rice polish the rats are able to make much greater gains in weight. It is thus seen that peanuts are relatively much richer in the anti-neuritic than in the pellagra-preventing fraction of vitamin B complex.

The rats made smaller gains when the peanuts were fed with the polyneuritic basal ration supplemented with an extract of rice polish

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## DEVELOPMENT OF CERTAIN STORAGE AND TRANSIT DISEASES OF CARROT<sup>1</sup>

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### INTRODUCTION

This paper deals in the main with four diseases of carrot (*Daucus carota* L.), namely, Sclerotinia soft rot (*Sclerotinia sclerotiorum* (Lib.) DBy),<sup>3</sup> Rhizopus soft rot (*Rhizopus tritici* Saito and *R. nigricans* Ehrenb.), bacterial soft rot (*Bacillus carotororus* L. R. Jones), and Botrytis rot (*Botrytis cinerea* Pers.)

No careful survey of the losses occasioned by these diseases in commercial storage has heretofore been reported. As judged by the reports of various workers and storage men, however, the losses are sometimes large, especially in cases of Sclerotinia soft rot and bacterial soft rot. Data are presented herein showing the losses due to a number of diseases under a variety of storage conditions.

Some idea of the losses caused by these diseases, incident to the shipment of topped carrots to market, may be obtained from Table 1. The data shown in this table were obtained from market-inspection certificates issued by the Bureau of Agricultural Economics, United States Department of Agriculture. The inspections upon which these certificates were based were made in response to a request from one of the parties interested—shippers, carriers, or receivers—and as a result of some question as to condition and grade. The certificates represent the total number of cars of topped carrots inspected from 1922 to 1927, inclusive. The distances covered in the shipment of these carrots ranged from a few miles to many hundred.

The 214 cars inspected represent only about 1 per cent of the 22,195 cars shipped during the same period. Although these shipments include both topped and untopped carrots, the percentage of topped carrots inspected is probably very small. The number of cars inspected is, of course, too small to give an adequate notion of the losses in the total shipment of carrots during this period. Since, however, the inspections were made over a period of years, some idea may be obtained of the occurrence and the relative importance of the several diseases.

<sup>1</sup> Received for publication Oct. 14, 1931, issued July, 1932.

<sup>2</sup> Formerly pathologist, Division of Horticultural Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>3</sup> The usual citation for this combination is (Lib.) Mass., but Miss Edith K. Cash, of the Bureau of Plant Industry, has found by searching the literature that apparently it was first used by De Bary in *Vergleichende Morphologie der Pilze*, p. 33, in 1884, 11 years prior to Massée's use of it.

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## APPARATUS AND EQUIPMENT

Most of the work was conducted in infection chambers (9)<sup>4</sup> or in storage rooms 8 feet wide, 14 feet long, and 11 feet high. The other equipment used is discussed in connection with the experiments in which it was employed.

## MATERIALS

## PATHOGENES

The pathogenes employed in the experiments were *Rhizopus tritici* isolated from sweetpotato (*Ipomoea batatas* (L.) Poir.), *R. nigricans* isolated from banana (*Musa paradisiaca* L.), *Sclerotinia sclerotiorum* isolated from cabbage (*Brassica oleracea* L.), *Botrytis cinerea* isolated from carrot, and *Bacillus carotovorus* isolated from carrot.

## HOSTS

The Danvers Half Long variety of carrot was used in the infection and storage experiments and in the temperature experiments that had as their object the determination of the cardinal temperatures for infection and decay. The following 18 varieties, with the exceptions noted in the text, were employed in the varietal-susceptibility tests and in some of the storage experiments: Blanche à collet vert (hors terre), Blanche lisse demi-longue, Carter Early Market, Carter Long Forcing, Carter Nantes, Carter Red Elephant, Carter Scarlet Perfection, Carter Summer Favorite, Danvers Half Long, Rouge demi-longue de Danvers, Jaune obtuse du Doubs, Rouge à forcer Parisienne, Rouge demi-courte de Guérande, Rouge demi-longue d'Amsterdam, Rouge demi-longue de Chantenay, Rouge demi-longue Nantaise, Rouge demi-longue de Saint James, and Rouge longue de Saint Valéry. The carrots were grown at the Arlington Experiment Farm, Rosslyn, Va., and stored, except when otherwise stated, at a temperature fluctuating between 0° and 2° C.

## SOURCES OF CONTAMINATION AND INFECTION

*Sclerotinia* soft rot differs from *Rhizopus* soft rot, bacterial soft rot, and *Botrytis* rot in the fact that its occurrence in storage depends upon field contamination and infection. This disease is common on carrots and a large number of other vegetable crops in the field. If the storage house has not been contaminated by the previous storage of vegetables affected with this disease, uncontaminated or uninfected roots from the field will not become infected by *S. sclerotiorum*.

Harvested carrots apparently are always contaminated with forms of *Rhizopus*, *Bacillus carotovorus*, and *Botrytis cinerea*, for infection by these pathogenes takes place whenever the roots are stored at certain temperatures and humidities. The sources of contamination are probably both the field and the storage house. All three of these organisms readily grow and reproduce on most, if not all, dead vegetable matter and may be expected to occur whenever such vegetable matter is present. *Bacillus carotovorus* frequently, and *B. cinerea* rarely, affect carrots in the field. In such cases contamination is traceable to field infection.

<sup>4</sup> Reference is made by number (italic) to Literature Cited, p. 911.

TABLE 1—Losses in 214 carloads of carrots, due to four storage diseases, from 1922 to 1927

Disease	Cars in which infection occurred		Average percentage of decay based on the number of—	
			Cars in which infection occurred	Cars inspected
	Number	Per cent		
Rhizopus soft rot.....	33	15.4	17	2.6
Sclerotinia soft rot.....	79	36.9	17	6.3
Bacterial soft rot.....	32	15.0	8	1.2
Botrytis rot.....	28	13.1	22	2.9
Soft rot <sup>a</sup> .....	15	7.0	8	.6

<sup>a</sup> The disease listed under this name may have been any one of the first three diseases

In some of the cars only one of the four diseases was found; in others, two or more. The average percentage of decay based on the 214 cars is suggestive of the total losses in carrot shipments. The average percentage based on the number of cars in which disease occurred gives a better picture of individual losses. The fact that these percentages are averages indicates that the individual losses varied, sometimes reaching 75 to 100 per cent. This unequal distribution of losses is a hopeful sign. The fact that there was no decay in many cars and slight decay in many others suggests that in these instances the factors favorable to decay were either eliminated or under partial control and that, if the same factors had been controlled or removed in these instances in which the losses were heavy, the total as well as the individual losses would have been smaller.

The data (Table 1) show that the greatest losses were caused by *Sclerotinia* soft rot, that those caused by *Rhizopus* soft rot and by *Botrytis* rot were about equal, and that those caused by bacterial soft rot were the smallest.

Many of the carrots had been stored before they were shipped and probably there were some losses due to decay. In preparing carrots for market it is the usual practice to sort out decayed roots. Losses resulting from sorting must therefore be added to those incurred during transit in computing total losses.

The data set forth herein are based on investigations that had as their object: (1) To make a survey of the diseases that normally affect carrots under a variety of storage conditions, (2) to determine the losses due to these diseases under different conditions of storage, (3) to determine the influence of temperature on the growth of some of the pathogens in culture media, (4) to study the influence of such factors as temperature, wounds, the presence or absence of organic matter, and the method of infection upon the decay of carrots by the four diseases mentioned above, (5) to test the susceptibility of 18 varieties of carrots to these diseases, and (6) to determine the conditions most favorable for the storage of carrots.

The work was done at the Arlington Experiment Farm, Rosslyn, Va., from 1920 to 1929, inclusive.

The results in Table 2 show that the unbroken skin of the carrot, if not a perfect barrier, is effective in preventing the entrance of this fungus. If the skin of the carrot root were continuous and uniform throughout, it would effectively limit the amount of decay caused by this pathogene. Unfortunately infection may readily occur at the openings of the tissue associated with the origin of the secondary roots and at the wounds that invariably result from handling in connection with the harvesting and storage of the crop.

#### WOUNDING AND INFECTION

In an experiment conducted to measure the influence of wounding at different temperatures, the wounded and unwounded roots were inoculated by dipping them in a suspension of mycelium and agar in water. The inoculum was prepared by squeezing carrot-agar cultures of *Sclerotinia sclerotiorum* through cheesecloth into a quantity of water. The wounding was accomplished by scraping off the skin of the roots, which gave a type of wound common in carrots, especially if roughly handled. A quantity of wounded and unwounded carrots were stored after inoculation at nine different temperatures. Except at one temperature (8° C.), the percentage of infection was larger in the wounded than in the unwounded roots (Table 3). Taking the results collectively at the temperatures at which infection occurred, there was 12 per cent more infection in the wounded than in the unwounded roots (32 as compared with 20 per cent). These data, together with those in Table 2, show that wounding is a factor of some importance in the infection and decay of carrots by *S. sclerotiorum*. Incidentally, the results at temperatures 11.5° and 12.5° C. (Table 3) indicate that the relative humidity of the storage chamber may also influence the amount of infection. Fresh wounding would have the effect of increasing the humidity at the wounded surface.

TABLE 3—*Infection of wounded and unwounded carrots stored in 12-quart baskets and inoculated with Sclerotinia sclerotiorum at various temperatures*

Temperature (° C.)	Relative humid- ity	Storage period	Wounded roots			Unwounded roots			Roots stored without treat- ment	
			Total	Infected		Total	Infected		Total	Infected
			Number ( <sup>a</sup> )	Number ( <sup>a</sup> )	Per cent ( <sup>a</sup> )	Number ( <sup>a</sup> )	Number ( <sup>a</sup> )	Per cent ( <sup>a</sup> )	Number ( <sup>a</sup> )	Per cent ( <sup>a</sup> )
33	93	20	107	0	0	84	0	0	80	0
26.5	91	20	160	18	11	84	2	2.4	84	0
24	93	20	107	28	26	84	15	18	110	0
19	95	20	90	46	51	109	26	24	117	0
18	95	20	101	69	68	75	20	27	99	0
12.5	96	40	121	81	67	106	61	58	89	0
11.5	96	40	109	7	6	88	10	11	103	0
8	96	40	103	6	6	110	0	0	91	0
7	96	40	97	0	0	104	0	0	82	0
3.5	92	40								
Total <sup>b</sup>			791	255	32	656	134	20	693	0

<sup>a</sup> All roots stored at 33° C. were decayed by Rhizopus.

<sup>b</sup> Totals obtained from the figures at the temperatures at which infection occurred.

## EXPERIMENTAL DATA

## SCLEROTINIA SOFT ROT

## THE PATHOGENE

Ramsey (12, 13) has shown that four species of *Sclerotinia* may cause decay of carrots under experimental conditions, namely, *S sclerotiorum*, *S intermedia* Ramsey, *S minor* Jagger, and *S ricini* Godfrey. He states that, of a large number of isolations made from vegetable products on the Chicago market during four years, more than 90 per cent yielded *S sclerotiorum*. He reports having isolated *S intermedia* from carrot only on two occasions (12, 13). So far as the writer is aware, *S minor* and *S ricini* have not been reported as normally causing decay of carrots. *S sclerotiorum*, therefore, is probably the principal species causing decay of carrots.

## METHOD OF INFECTION

Boyle (1) asserts in his work on the Scarlet Runner bean (*Phaseolus coccineus* Jacq.) and broadbean (*Vicia faba* L.) that *Sclerotinia sclerotiorum* obtains entrance to the host tissue by mechanical pressure and that it has the ability to penetrate the cuticle as well as the subcuticular layers. He did not, however, test such tissues as those in the roots of carrots. In the present experiments no effort was made to determine the method by which the pathogene gains entrance to the cells of the carrot, but two experiments (Table 2) were conducted to determine whether there is a normal barrier to the entrance of the fungus into the host tissue.

In these experiments glass tubes 5 mm in diameter and 10 mm long were sealed over fresh wounds, old wounds, areas where secondary rootlets emerge from the primary roots, and the uninjured skin. The tubes were then filled, in the first experiment with sweetpotato decoction, and in the second with carrot decoction, and bits of the mycelium of *Sclerotinia sclerotiorum* were introduced into each tube. The fresh wounds were made by cutting off a slice of tissue from the roots with a sharp knife. The old wounds consisted of areas where the skin had been rubbed or scratched off in the process of harvesting and storing. The roots had been in storage a little over four months in each case. When the roots were to be inoculated over the uninjured skin considerable care was exercised to select areas free from injury. After inoculation the roots were stored at 15° C., for 22 days in the first experiment and for 42 days in the second experiment.

TABLE 2—Infection of carrots inoculated with *Sclerotinia sclerotiorum* over fresh wounds, old wounds, rootlets, and the unbroken skin, and held at a temperature of 15° C.

Inoculation over—	Storage period		Roots used	Roots infected	
	Experiment 1 <sup>a</sup>	Experiment 2 <sup>b</sup>			
	Days	Days	Number	Number	Per cent
Fresh wounds.....	22	42	12	10	83
Old wounds.....	22	42	12	10	83
Rootlets.....	22	42	13	4	31
Unbroken skin.....	22	42	23	0	0

<sup>a</sup> The inoculum consisted of bits of the mycelium of *S sclerotiorum* in sweetpotato decoction.

<sup>b</sup> The inoculum consisted of bits of the mycelium of *S sclerotiorum* in carrot decoction.

The results recorded in Table 5 were obtained from an experiment in which bits of sclerotia were used as the inoculum. The roots were thoroughly washed, a small piece of sclerotium (about 0.5 mm thick by 1 mm square) was inserted in the thickest diameter of each, and the roots were stored at the various temperatures in wire baskets 12 inches in diameter and 12 inches deep.

TABLE 5—*Influence of temperature on infection and decay of carrots inoculated with Sclerotinia sclerotiorum*

[Bits of sclerotia about 0.5 mm thick by 1 mm square were used as inoculum]

Temperature (° C)	Storage period	Roots used	Roots infected		Average area of lesions	Temperature (° C)	Storage period	Roots used	Roots infected		Average area of lesions
			Number	Per cent					Number	Per cent	
32.....	6	30	0	0	0	10.....	6	31	0	0	0
28.....	6	30	22	73	223	14.....	11	33	32	97	295
25.5.....	6	33	30	91	474	12.....	11	34	28	82	284
23.....	6	34	28	82	541	10.....	11	31	20	65	58
20.....	6	33	30	91	504	5.....	11	31	0	0	0
19.5.....	6	33	32	97	463	5.....	41	31	9	29	283
14.....	6	33	16	48	35	3.5.....	41	34	2	6	1.6
12.....	6	34	8	24	11						

The highest temperature at which infection occurred in six days was 28° C. It is not possible to hold carrots at temperatures above 28° except for a short period because of contamination and infection by other fungi. Ramsey (13) obtained negative results when young carrot roots were inoculated with *Sclerotinia sclerotiorum* and exposed to a temperature fluctuating between 31° and 33° and averaging 32°. The maximum temperature for infection would therefore seem to be slightly lower than that for growth on culture media. Maximum decay was obtained at a temperature of 23° (Table 5), which corresponds closely to the optimum temperature (24°) for growth on carrot agar (Table 4.) The discrepancy of 1° may be accounted for by the different temperatures employed in the two cases. The rate of decay declined rapidly as the temperature rose above or fell below 23°.

The lower temperature limit for infection has not been accurately determined, but it is sufficiently near the lower temperature limit for the storage of carrots to make its elimination by the manipulation of temperature impracticable. Ramsey (13) has found that *Sclerotinia sclerotiorum* will infect bean pods at a temperature of 0° C. Infection of carrots with this fungus has been obtained at temperatures ranging from 0° to 1° (Table 7.) Carrots can not be stored at temperatures much below 0° without danger of freezing, the freezing point being about -1.4° (15).

If carrots contaminated with this pathogene are stored, it is possible to check the losses by holding the temperature near 0° C., but it is not possible entirely to eliminate decay. The control of this disease should begin in the field.

## INFLUENCE OF TEMPERATURE ON GROWTH OF THE PATHOGENE ON CULTURE MEDIA

The fungus was grown on carrot agar in 200-cc Erlenmeyer flasks. The flasks were inoculated by introducing into each, with a sterile needle, a small piece of agar containing mycelium from a pure culture. About the same amount of inoculum was used in each flask. Ten flasks were incubated at each temperature. As each lot was inoculated the flasks were placed immediately at the various temperatures. The surface area of the fungus colony was used as a measure of the amount of growth.

The highest temperature at which *Sclerotinia sclerotiorum* was observed to grow was 32.5° C (Table 4). No growth took place in 3 days at 35°. This maximum temperature for growth corresponds closely with that obtained by Ramsey (13) on potato-dextrose agar, where it grew very slowly at 32° and 33°. Maximum growth occurred at 24° after 3 days (Table 4). A slight amount of growth occurred at 0.9° in 22 days in 2 flasks out of 10. No observations were made at temperatures below 0.9°. Growth declined rapidly as the temperature rose above or fell below 24°. (Table 4)

TABLE 4—Influence of temperature on the growth of *Sclerotinia sclerotiorum* on carrot agar in Erlenmeyer flasks

Temperature (° C)	Period of exposure	Colonies measured	Average area of colonies	Temperature (° C)	Period of exposure	Colonies measured	Average area of colonies
	Days	Number	Mm <sup>2</sup> *		Days	Number	Mm <sup>2</sup> *
35.....	3	0	0	12.....	3	0	0
32.5.....	3	(b)	0	12.....	10	9	2, 04b
30.....	3	10	1, 017	7.5.....	10	(c)	0
26.5.....	3	10	2, 442	5.5.....	10	0	0
24.....	3	10	3, 064	7.5.....	22	(d)	0
23.5.....	3	10	2, 430	5.5.....	22	10	535
21.5.....	3	10	1, 697	2.....	22	(e)	0
19.....	3	10	773	9.....	22	(f)	0
15.5.....	3	10	309				

\* Mm<sup>2</sup> is the abbreviation for square millimeter recently adopted for U. S. Government printing.

<sup>b</sup> Growth just started in 1 flask.

<sup>c</sup> Growth just started.

<sup>d</sup> Growth covered flasks.

<sup>e</sup> Growth just started in 2 flasks.

## INFLUENCE OF TEMPERATURE ON INFECTION AND DECAY

To obtain a uniform amount of inoculum for all the roots used in a given experiment and at the same time to confine the initiation of infection to a definite area, so that a comparable quantitative measurement may be made of the decay produced at different temperatures, is more difficult with a fungus like *Sclerotinia sclerotiorum*, which does not normally fruit on culture media, than with fungi that sporulate abundantly. Bits of mycelium, no matter how obtained, are bound to vary. The same is true of bits of sclerotia, although a more uniform quantity of inoculum can be obtained by carefully slicing the sclerotia. For comparisons of the effect of temperature on the amount of decay, both mycelium and sclerotia have been found fairly satisfactory if large numbers of roots are used to eliminate the effects of individual variation. Mycelium inoculation yields a larger percentage of infection, but fairly young sclerotia also yield a large percentage.

influenced by two factors (1) The greater amount of water present on the undried roots at the outset of the experiment and (2) the loss of some of the inoculum from the dried roots, due to the drying process and the handling incident to it, although the roots were handled carefully to avoid this. That this pathogene is sensitive to the amount of moisture present is shown by the reduced percentage of infection in the wet roots at a relative humidity of 80 per cent as compared with that at 90 per cent, as well as by the similar effect of the relative humidity on infection in the dried roots.

#### VARIETAL SUSCEPTIBILITY TO INFECTION AND DECAY

To measure the relative susceptibility of 14 varieties of carrots to infection by *Sclerotinia sclerotiorum*, three types of inoculum, two methods of inoculation, and three criteria of measurements were employed.

The three types of inoculum consisted, respectively, of sclerotia, mycelium, and carrots decaying with *Sclerotinia sclerotiorum*. The method of inoculation with either sclerotia or mycelium was to insert, by means of a small scalpel, bits of the inoculum as nearly the same size as possible one-fourth to one-eighth of an inch deep in the thickest diameter of the roots. The method of inoculation with the third type of inoculum was to place near the center of a quantity of carrots one decaying with the pathogene. The percentage of roots infected and the diameter of the lesions were used as measures of susceptibility in the experiments in which the roots were inoculated with bits of mycelium or sclerotium, the percentage of infection and the diameter of the nests that developed were the criteria of susceptibility when the carrots were inoculated with a decaying carrot. There was one replication of each experiment involving each type of inoculum.

The conclusions drawn from all the data obtained are (1) That all the varieties tested are readily susceptible to decay by *Sclerotinia sclerotiorum*, (2) that, although there is some variation in the number of infections, the percentage of infection, and the degree of decay with the different varieties in a given experiment, these variations are not always paralleled in another experiment conducted as nearly as possible under the same conditions, and (3) that the amount of variation is probably not always a measure of relative susceptibility but is due rather to uncontrollable factors, such as kind and quantity of inoculum, size and shape of roots, variation in the viability of the inoculum, variation in susceptibility within a host variety, degree of wounding, and probably some unknown factors.

The results recorded in Table 7 are submitted as an example of the relative susceptibility of 12 varieties at three different temperatures. The conclusions drawn from these data apply also to the two varieties Rouge demi-longue de Saint James and Rouge longue de Saint Valery, used in some of the other experiments but not in the present one. In this experiment the roots were inoculated by placing a decaying carrot in the center of a 12-quart wire basket of roots of each variety. All the roots were as nearly the same size as possible, and only sound roots that were fairly free from blenushes were used. Considering that in this experiment only one inoculation is involved in the case of each variety at each temperature and that variation in the size and shape of the roots and irregularity in the pack-

RELATION OF HUMIDITY TO INFECTION<sup>5</sup>

Ramsey (13) states that moisture is an important factor in the infection of vegetables by *Sclerotinia sclerotiorum*. However, he presents no data on the influence of air humidity on infection.

In an experiment (Table 6) conducted for the purpose of studying the influence of humidity on infection, carrots (Danvers Half Long) grown in the vicinity of Canton, Pa., were used. The roots were obtained directly from the field, stored a few days in a cool basement, and incorporated in the experiment November 30, 1925. Only sound roots and roots relatively free from wounds were used. The roots were stored in chambers 7 by 9 by 10 feet high, provided with ventilation (14), and all four chambers were maintained at a temperature of 6.5° C. The relative humidities of these rooms were 95, 90, 80, and 70 per cent, respectively. One 16-quart hamper of untreated roots was placed in each room as checks. Six hampers of carrots were inoculated with *Sclerotinia sclerotiorum* by dipping them in a suspension of mycelium and carrot agar in water. The suspension was made by squeezing six 200-cc Erlenmeyer flasks of carrot-agar cultures (40 cc to the flask) through fine-mesh cheesecloth into 10 gallons of water. After inoculation the roots from four of the hampers were spread out carefully on the floor of a large room and dried with an electric fan. As soon as dry, one hamper of roots was placed in each of the four storage chambers. The two remaining hampers were placed without drying at relative humidities of 90 and 80 per cent, respectively.

In the checks at 95 per cent relative humidity two roots became infected, indicating that there was some contamination of field origin.

TABLE 6—Influence of humidity on infection of carrots inoculated with *Sclerotinia sclerotiorum* and stored at 6.5° C

Relative humidity (per cent)	Storage period	Inoculated roots						Untreated roots		
		Stored without drying			Stored after drying			Total	Infected	
		Total		Infected	Total		Infected			
		Number	Number		Per cent	Number				
95.....	47				41	4	10	33	2	6
90.....	47	37	7	19	44	1	2	37	0	0
80.....	47	42	3	7	39	0	0	38	0	0
70.....	47				42	0	0	40	0	0
95.....	104				41	12	29	33	2	6
90.....	104	37	24	65	44	2	5	37	0	0
80.....	104	42	10	24	39	0	0	38	0	0
70.....	104				42	0	0	40	0	0

Infection resulting from inoculation was much more marked in the roots stored in the wet condition than in the roots stored after they were dried. Not only was the percentage of infection higher in the former, but infection developed at a relatively lower percentage of humidity (80 per cent). This increased infection may have been

<sup>5</sup> The results recorded in Tables 6, 27, and 35 were obtained from an experiment conducted at the Marble Laboratory (Inc.), Canton, Pa., through the courtesy of S. M. Marble, who was responsible for maintaining the temperature, humidity, and ventilation throughout the experiment. The results discussed regarding the effect of humidity on infection of carrots by *Bacillus carotovorus* also were obtained in connection with this experiment.

somewhat, depending on the particular lot of carrots. As a rule the amount of infection is relatively small below 30°. Between 20° and 25°, the number of infections is probably influenced by the infection and decay produced by *Bacillus carotovorus*, which is often heavy. The absence of *Rhizopus* infection at 24° in experiment 2, Table 8, may easily be accounted for by the *B. carotovorus* infection, which amounted to 94 per cent at the end of 19 days. Infection by *Rhizopus* at temperatures below 20° is erratic in its occurrence, often not appearing at all during the marketable life of the roots and never in large amounts. In only two instances during nearly 10 years of experience in the storage of carrots has the writer observed infection of uninoculated carrots by *Rhizopus* at temperatures below 12°. At temperatures above 30°, infection by *Rhizopus* is heavy and occurs in a very short time.

TABLE 8—*Influence of temperature on infection of carrots by Rhizopus*

## EXPERIMENT 1

Temperature (° C)	Relative humidity	Duration of storage	Inoculated roots <sup>a</sup>			Untreated roots		
			Total	Infected		Total	Infected	
	Per cent	Days	Number	Number	Per cent	Number	Number <sup>(b)</sup>	Per cent
44.....	92	11	54	6	11	66	59	76
41.....	92	11	70	( <sup>b</sup> )	( <sup>b</sup> )	86	( <sup>b</sup> )	43
35.....	87	11	74	64	86	78	3	4
32.....	78	11	62	44	71	60	26	43
24 5.....	96	22	58	1	2	70	3	4
22 5.....	93	22	79	0	0	58	1	2
20.....	97	22	61	1	2	81	0	0
18.....	96	22	60	1	2	69	0	0
14 5.....	95	31	69	0	0	84	0	0
11 5.....	95	31	59	0	0	65	0	0
10.....	98	31	53	0	0	56	0	0
8.....	90	31	67	0	0	74	0	0
5.....	100	31	48	0	0	66	0	0
3.....	100	31	65	0	0	71	0	0

## EXPERIMENT 2 (UNTREATED ROOTS)

Temperature (° C)	Relative humidity	Duration of storage	Total roots	Roots infected by—			
				R. tritici		R. nigricans	
	Per cent	Days	Number	Number	Per cent	Number	Per cent
36 5.....	93	12	43	35	81	0	0
31.....	91	19	48	9	19	0	0
24 5.....	93	27	48	4	8	0	0
24.....	95	19	57	0	0	0	0
21 5.....	95	27	59	5	8	0	0
19.....	92	27	50	3	6	2	4
15 5.....	91	51	63	0	0	5	8
12 5.....	95	51	48	0	0	2	4
8.....	91	79	47	0	0	0	0
6 5.....	95	113	45	0	0	0	0
2.....	94	113	45	0	0	0	0
1.....	91	113	61	0	0	0	0

<sup>a</sup> The inoculum consisted of a spore suspension of *R. tritici* and *R. nigricans*.

<sup>b</sup> Infection by *Penicillium* was so great at temperatures of 41° and 44° C. as to make a count of *Rhizopus* infection uncertain, except in the case given.

## EFFECT OF TIME

The results recorded in Table 9 were compiled from data obtained in connection with experiment 2, Table 8, to show the effect of time on infection. The period of time employed was governed not only

ing must of necessity exist in the different baskets, the results obtained in the number of infections, in the percentage of infection, and in the diameters of the nests are regarded as unusually uniform. Certainly all the varieties are readily susceptible and if there is any difference in their susceptibility it is of no practical consequence. The results of experiments previously described confirm this conclusion.

TABLE 7.—Infection of 12 varieties of carrots inoculated with *Sclerotinia sclerotiorum* and stored at temperatures of 10°, 4.5°, and 0° to 1° C for 92, 92, and 155 days, respectively

Variety	Infection after—													
	92 days at 10°						92 days at 4 5°			155 days at 0° to 1°				
	Roots used	Roots infected		Extent of infection	Roots used	Roots infected		Diameter of nest	Roots used	Roots infected		Diameter of nest		
No	No	P ct	No	No	P ct	Mm	No	No	P ct	Mm				
Carter Early Market.....	34	21	62	Infection through-out basket	46	15	33	112	39	4	10	40		
Carter Long Forcing.....	65	60	92	do.....	75	16	21	76	66	4	6	40		
Carter Nantes.....	52	34	65	Infection through-out basket	76	19	25	108	63	7	11	50		
Carter Red Elephant.....	52	34	65	do.....	52	12	23	127	65	7	11	50		
Carter Scarlet Perfection.....	44	42	95	do.....	52	19	37	108	43	6	14	55		
Carter Summer Favorite.....	52	52	100	do.....	52	19	37	112	55	2	4	45		
Danvers Half Long.....	61	44	72	do.....	66	18	27	102	71	6	8	35		
Rouge à forcer Parisienne.....	79	75	95	do.....	69	28	41	112	74	3	4	45		
Rouge demi-courte de Guérande.....	56	55	98	do.....	84	24	29	102	71	4	6	50		
Rouge demi-longue d'Amsterdam.....	53	45	85	do.....	68	32	47	140	55	6	11	70		
Rouge demi-longue de Chantenay.....	43	39	91	do.....	48	23	48	133	57	4	7	50		
Rouge demi-longue Nantaise.....	53	48	91	do.....	55	19	35	112	50	4	8	55		

#### RHIZOPUS SOFT ROT

##### SPECIES OF RHIZOPUS RESPONSIBLE FOR DECAY

*Rhizopus tritici*<sup>6</sup> and *R. nigricans* are the only species of *Rhizopus* isolated from carrots by the writer. Other species may cause decay (3) under special conditions, but since these two species are the only ones obtained from a large number of isolations, it is believed that they are the chief if not the only species that cause decay.

##### FACTORS AFFECTING DISTRIBUTION OF RHIZOPUS INFECTION AT DIFFERENT TEMPERATURES

###### DISTRIBUTION IN UNTREATED ROOTS

The results recorded in Table 8 on the distribution of *Rhizopus* at different temperatures are representative (except for the qualifications given in the text) of experiments conducted during different times of the year for a number of years.

If sound, untreated carrots, directly from the field or after having been stored at 0° to 2° C for a time, are stored at different temperatures from 0° to 44°, infection by forms of *Rhizopus* usually occurs at about 15° to 44°. (Table 8.) The lower limit of this range varies

<sup>6</sup> The specific name *tritici* (9) as here used may easily include *tritici* itself, *Rhizopus nodosus* Namys, *R. oryzae* Went and Pr Gerlugs, and *R. delemar* (Boid) Wehmer and Hanzawa, for no morphological characterizations are available by which these species can be definitely separated.

TABLE 10—*Infection at various temperatures of wounded and unwounded carrots by Rhizopus*—Continued

## ROOTS NOT INOCULATED

Temperature (°C)	Storage period	Wounded roots				Unwounded roots			
		Total	In-fected	Isola-tions	Organism isolated	Total	In-fected	Isola-tions	Organism isolated
	Days	Number	Number	Number		Number	Number	Number	
32.....	20	15	4	4	<i>R. tritici</i> .....	15	6	6	<i>R. tritici</i>
25.....	20	15	0	0	.....	15	0	0	.....
19.....	32	15	0	0	.....	15	0	0	.....
8.....	32	15	0	0	.....	15	0	0	.....
5.5.....	32	15	0	0	.....	15	0	0	.....

Inoculation by dipping in a spore suspension sometimes seems to lower the temperature limit for infection. In the roots in experiment 1, Table 8, inoculated with *Rhizopus nigricans* and *R. tritici*, and in roots (only the unwounded roots are considered in this comparison) inoculated with *R. tritici* alone (Table 10), infection was obtained at a slightly lower temperature than in the uninoculated roots. On the other hand, in roots inoculated with *R. nigricans* alone and with *R. tritici* plus *R. nigricans* the lowest temperature, 32° C (Table 10), at which infection occurred was the same as in the uninoculated roots. Infection has even occurred at a lower temperature, 12.5°, in uninoculated roots in one experiment (Table 9) than in inoculated roots in another, 18° (experiment 1, Table 8). Aside from the difference in the history of the two lots, there is some difference in the temperatures and periods of time employed, thus making a direct comparison impracticable. The foregoing results justify the conclusion that natural inoculation, so far as the lower temperature limit for infection is concerned, is not a limiting factor in some lots of carrots stored directly from the field, and emphasize the uncertainty of *Rhizopus* infection at temperatures below 20°. By employing the extreme conditions involved in the "well" method of inoculation<sup>7</sup> (4), it is possible to alter either the normal limits of infection by *R. tritici* and *R. nigricans* or the limits obtained by inoculation with a spore suspension. The lower temperature limits of infection by both pathogens may thus be lowered and the upper limit of *R. nigricans* raised, until the temperature limits of infection are almost as wide as the temperature limits of growth. The results obtained by this method of inoculation will be discussed later.

## EFFECT OF WOUNDING

The data recorded in Table 10 are from one of three experiments conducted to determine the effects of wounding on the infection of carrots by *Rhizopus* at different temperatures. A different type of wounding was employed in each experiment: (1) Scraping the skin off the root with a knife, (2) striking the root on the side of a wire basket, and (3) cutting a small slice off each root. The last-named type of wounding was used in the experiment reported. In the unreported experiments the wounded and unwounded roots were inoculated by dipping them in a mixed spore suspension of *Rhizopus tritici*.

<sup>7</sup> The well method of inoculation consists in placing a 24 to 48 hour old test-tube culture of the organism on liquid medium in a "well" in a root one-half to 1 inch deep. This is made with a 1/4-inch cork borer and the opening is plugged with cotton after inoculation.

by infection by *Rhizopus* but also by infection by *Bacillus carotovorus* and forms of *Penicillium* and *Fusarium*, which tends to complicate the problem and to make the selection of comparable periods impracticable. The results recorded in Tables 8 and 9 show quite clearly that carrots are much more resistant to infection by *Rhizopus* at temperatures below 30° C than above it.

TABLE 9—Influence of time and temperature on normal infection of carrots by *Rhizopus*

Temperature (°C )	Relative humidity	Roots used	Roots infected by Rhizopus after—								
			4 days	5 days	6 days	8 days	12 days	19 days	27 days	51 days	
	Per cent	Number	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	
36.5.....	93	43	26	44	56	70	81	-----	-----	-----	
31.....	91	48	2	4	4	4	15	19	-----	-----	
24.5.....	93	48	0	0	0	0	0	4	8	-----	
21.5.....	88	39	0	0	0	0	2	3	8	-----	
19.....	92	50	0	0	0	0	0	2	10	-----	
15.5.....	91	63	0	0	0	0	0	0	8	8	
12.5.....	95	48	0	0	0	0	0	0	0	4	

The separate distribution of *Rhizopus tritici* and *R. nigricans* will be discussed later.

#### EFFECT OF INOCULATION

If carrots are inoculated by being dipped in a spore suspension of *Rhizopus tritici* and *R. nigricans*, the percentage of infection in a given period is usually greater than that of uninoculated roots (Table 8, experiment 1, and Table 10).

TABLE 10—Infection at various temperatures of wounded and unwounded carrots by *Rhizopus*

ROOTS INOCULATED WITH <i>R. TRITICI</i>									
Temperature (°C)	Storage period	Wounded roots				Unwounded roots			
		Total	In-fected	Isola-tions	Organism isol. ted	Total	In-fected	Isola-tions	Organism isolated
	Days	Number	Number	Number		Number	Number	Number	
32.....	20	15	14	12	<i>Rhizopus tritici</i> .....	15	14	9	<i>R. tritici</i>
25.....	20	15	5	5	do.....	15	1	1	Do
19.....	32	15	1	0	.....	15	1	1	Do
15.5.....	32	15	0	0	.....	15	0	0	
8.....	32	15	0	0	.....	15	0	0	
5.5.....	32	15	0	0	.....	15	0	0	

ROOTS INOCULATED WITH <i>R. NIGRICANS</i>									
32.....	20	15	10	8	<i>Rhizopus tritici</i> .....	15	5	3	<i>R. tritici</i>
25.....	20	15	4	0	.....	15	0	0	
19.....	32	15	1	0	.....	15	0	0	
15.5.....	32	15	0	0	.....	15	0	0	
8.....	32	15	0	0	.....	15	0	0	
5.5.....	32	15	0	0	.....	15	0	0	

ROOTS INOCULATED WITH <i>R. TRITICI</i> PLUS <i>R. NIGRICANS</i>									
32.....	20	15	11	9	<i>R. tritici</i> .....	15	15	10	<i>R. tritici</i>
25.....	20	15	1	1	do.....	15	0	0	
19.....	32	15	1	1	<i>R. nigricans</i> .....	15	0	0	
8.....	32	15	0	0	.....	15	0	0	
5.5.....	32	15	0	0	.....	15	0	0	

into wells made in the thickest diameter of the roots, and others by using cultures of *R. tritici* of the same age and grown under the same conditions as the former species. The wells were plugged with sterile cotton and the roots then exposed to the various temperatures. After decay had advanced sufficiently, the carrots were weighed, the decay removed, and the undecayed portion weighed again. The amount of decay at each temperature was obtained by subtracting the second weight from the first.

A number of experiments were made with each pathogene, the results of which are recorded in Tables 11 and 12.

TABLE 11—*Influence of temperature on infection and decay of carrots inoculated with Rhizopus tritici*

[20 roots used in all cases]

Temperature (°C)	Storage period	Roots in- fected	Decay	Temperature (°C)	Storage period	Roots in- fected	Decay
	Days	Number	Grams		Days	Number	Grams
35.5.....	2	20	283	18.5.....	2	20	56
33.5.....	2	20	388	15.....	2	20	26
32.....	2	20	341	11.5.....	2	20	11
30.....	2	20	335	9.5.....	2	20	8
28.....	2	20	218	8.....	2	0	0
24.5.....	2	20	161	8.....	7	20	51
23.....	2	20	121	5.....	7	4	6
20.....	2	20	80	2.5.....	7	0	0

TABLE 12—*Influence of temperature on infection and decay of carrots inoculated with Rhizopus nigricans*

[20 roots used in all cases]

Temperature (°C)	Storage period	Roots in- fected	Decay	Temperature (°C)	Storage period	Roots in- fected	Decay
	Days	Number	Grams ( <sup>a</sup> )		Days	Number	Grams ( <sup>b</sup> )
35.....	2	4		18.5.....	2	20	249
33.5.....	2	20	91	13.....	2	20	150
31.5.....	2	20	292	12.....	2	20	34
28.5.....	2	20	420	9.5.....	2	20	22
28.....	2	20	482	8.....	2	10	
24.5.....	2	20	465	5.....	2	0	0
22.5.....	2	20	419	5.....	13	0	0
20.....	2	20	225	2.....	13	0	0

<sup>a</sup> Just started. Decay at this temperature was due to *Rhizopus tritici*.

<sup>b</sup> Six just started.

It will be seen that both *Rhizopus tritici* and *R. nigricans* are capable of infecting and decaying carrots over a far wider range of temperatures under the conditions of these experiments than under the conditions of the preceding experiments. The obvious differences in the factors operating in the two sets of experiments are the degree of wounding; the amount of inoculum in a given area, and the presence of a medium, in the form of carrot decoction, in the wells in the latter experiments as compared with its absence in the former. An enzyme<sup>8</sup> also may have been present in the carrot decoction to act on the carrot tissue ahead of infection.

It is believed that the degree of wounding was not a limiting factor in the earlier experiments, for carrots have often been subjected to

<sup>8</sup> Observations have shown that the action of *Rhizopus tritici* and *R. nigricans* is the same on carrots as on sweetpotatoes (2). This action consists largely in dissolving the middle lamellae through the agency of pectinase secreted by the pathogenes.

and *R. nigricans*. In the present experiment three spore suspensions were used: *R. tritici* alone, *R. nigricans* alone, and a mixed suspension of the two pathogenes.

The suspensions were prepared by pouring water on cultures of the pathogenes in Erlenmeyer flasks, shaking the flasks to free the spores, and then pouring the suspension through cheesecloth into a larger volume of water to remove as far as possible the mycelium present. The three spore suspensions were made up separately, so that the dosages are not necessarily comparable in the lots inoculated with the different suspensions; but they are comparable in the wounded and unwounded roots inoculated with a given inoculum. The checks consisted of uninoculated wounded and unwounded roots.

At temperatures of 19°, 25°, and 32° C., the only temperatures at which any infection occurred (Table 10), 52, or 29 per cent, of the 180 wounded roots became infected, as compared with 42, or 23 per cent, of the 180 unwounded roots. The margin of infection in the wounded over the unwounded roots was not large (6 per cent), and in two cases the percentage of infection was greater in the unwounded lots.

The results of the three experiments show a somewhat greater percentage of infection in the wounded than in the unwounded carrots, there being exceptions in particular lots. In some instances infection occurred at lower temperatures in wounded than in unwounded roots.

#### TEMPERATURES AT WHICH RHIZOPUS NIGRICANS AND *R. TRITICI* PRODUCE INFECTION

From the data recorded in Table 8 (experiment 2) and Table 10, it appears that 19° C. is near the dividing line between temperatures at which infection is produced by *Rhizopus tritici* on the one hand and *R. nigricans* on the other. *R. tritici* was obtained in all the isolations made from infected roots stored at temperatures above 19°, while *R. nigricans* was obtained from infected roots stored below 19°. These results were confirmed by another experiment made under the same conditions as experiment 2, Table 8.

The foregoing results indicate that, although the amount of inoculum on stored carrots as well as the presence of wounds may increase or otherwise influence infection by *Rhizopus tritici* and *R. nigricans*, artificial inoculation and wounding are not essential to infection. Carrots are very susceptible to decay at temperatures above 30°, much less susceptible between 20° and 30°, and highly resistant below 20°. Carrots are highly resistant to attack by *R. nigricans* under all circumstances.

#### EFFECT OF TEMPERATURE ON INFECTION AND QUANTITY OF DECAY

The well method was employed to determine the temperature ranges at which *Rhizopus tritici* and *R. nigricans* are able to infect carrots, and to measure the influence of temperature on the quantity of decay during certain periods of time.

Roots of the Danvers Half Long variety were washed in soapy water, rinsed in tap water, and dried in the laboratory. Some were inoculated by introducing 48-hour-old cultures of *Rhizopus nigricans*, grown in 2.5 c.c. of carrot decoction in test tubes at room temperature,

*R. nigricans* normally causes all the decay *R. tritici* causes all the decay of sweetpotatoes above 32°, and the infection is divided between the two species at temperatures between 20° and 32° (6) The normal range of temperature at which *R. tritici* infects wounded and unwounded carrots extends from about 19° to 44°, and the range at which it infects wounded sweetpotatoes is from 20° to 44° (7) Wounded sweetpotatoes at temperatures below 26° are very susceptible to infection by *R. nigricans*, whereas wounded or unwounded carrots are highly resistant to infection by this pathogene (8) The resistance of sweetpotatoes to infection by *Rhizopus* resides in the skin and healed-over wounded surfaces, and fresh wounding removes this resistance (11), whereas the resistance of carrots to infection by *Rhizopus* is affected very little by wounding The normal resistance of sweetpotatoes to infection by *R. tritici* at temperatures below 20° is broken down when the roots are inoculated by the well method, the resistance of carrots to *R. tritici* at temperatures below 20° and to *R. nigricans* at temperatures from 8° to 33.5° is broken down when the roots are inoculated by the well method

#### VARIETAL SUSCEPTIBILITY TO DECAY

##### SUSCEPTIBILITY TO RHIZOPUS NIGRICANS

It has already been shown that either the Danvers Half Long variety is normally resistant to infection and decay by *Rhizopus nigricans* or that this pathogene is not well equipped to infect the roots under normal conditions The following varieties of carrot have been found to be susceptible when inoculated by the well method with 24-hour to 48-hour cultures grown in 2.5 c.c. of carrot decoction in test tubes, plugged with cotton, and stored at temperatures fluctuating from 10° to 15° C. Blanche à collet vert (hors terre), Blanche lisse demi-longue, Carter Early Market, Carter Long Forcing, Carter Nantes, Carter Red Elephant, Carter Scarlet Perfection, Carter Summer Favorite, Danvers Half Long, Rouge demi-longue de Danvers, Jaune obtuse du Doubs, Rouge à forcer Parisienne, Rouge demi-courte de Guérande, Rouge demi-longue d'Amsterdam, Rouge demi-longue de Chantenay, Rouge demi-longue Nantaise, and Rouge longue de Saint Valery. The degree of susceptibility does not seem to vary greatly under these conditions

During one to four different seasons all these varieties were stored at temperatures ranging from 0° to 15.5° C. The only infection recorded, aside from that of Danvers Half Long by *Rhizopus*, was on Rouge demi-longue de Danvers held at a temperature of 10° during the season of 1926-27, when about 5 per cent of the stock stored (a little more than a peck) was decayed This variety was stored the two preceding seasons without infection by *Rhizopus* This variety is really a strain of Danvers Half Long, and in general there has been found very little difference between the two in their reaction to disease Although no isolations were made from these roots, because they were completely decayed at the time of the inspection, it is believed, because of earlier experience, that the decay was due to *R. nigricans* In any case, the varieties listed above probably can safely be stored at temperatures below 15° without much danger of infection by *R. nigricans*.

various degrees of wounding, dipped in a spore suspension of either species of *Rhizopus*, and exposed to temperatures below 20° C, with very little resulting infection.

The presence of a greater quantity of inoculum and culture medium in the wells seems to be the factor operating in the present experiments to overcome the resistance of the roots at temperatures at which they are normally resistant.

By the end of two days the amount of decay caused by *Rhizopus tritici* (Table 11) increased from 0 g at 8° C to 388 g at 33.5° and decreased progressively as the temperature rose above 33.5°. In a supplementary experiment, the maximum amount of decay was obtained at 35.5° and declined as the temperature rose. Some decay occurred at 42°. At 44° the roots showed evidence of injury and were badly infected with *Penicillium*. Active decay has been found a number of times at a temperature as low as 8°, and *R. tritici* has been isolated from the decay at this temperature. Below 8°, if decay begins at all, it tends to dry up. The lowest temperature at which decay by *R. tritici* has been observed is 5°. This minimum is slightly higher than that (3.5°) observed in sweetpotatoes (9).

After two days the amount of decay produced by *Rhizopus nigricans* (Table 12) increased from 0 g at 5° C to a maximum of 482 g at 28° and decreased progressively as the temperature rose above 28°. It was not possible to determine the maximum temperature for infection of carrots by *R. nigricans*, because of the invasion of *R. tritici*, which infects carrots readily at a temperature of about 35°. In fact it is quite probable that some of the decay at 31.5° and 33.5° was due to *R. tritici*. A large percentage of the isolations made from carrots inoculated as in this experiment have yielded *R. tritici*. In the present case *R. tritici* alone was obtained at 35°. Isolations were not made from roots stored at the other temperatures. The lowest temperature at which decay by *R. nigricans* has been found is 8°.

#### RHIZOPUS INFECTION IN CARROTS AND SWEETPOTATOES

A comparison of infection of carrots and sweetpotatoes (9, 10) by species of *Rhizopus* brings out some interesting differences and similarities.

(1) Fresh wounds are almost invariably essential to infection of sweetpotatoes at temperatures below 33° C. Some infection will occur without wounding above 33°. Wounds have very little effect on infection of carrots at any temperature. (2) Some change takes place in sweetpotatoes when exposed to temperatures above 33° that makes them fairly susceptible to decay without wounding. Some change takes place in carrots when exposed to temperatures above 30° that makes them very susceptible to decay. (3) *Rhizopus tritici* and *R. nigricans* cause most if not all the decay in both cases. (4) The total temperature range at which *Rhizopus* has been found to infect carrots (0°–2° to 44°) and that at which it infects sweetpotatoes (3.5° to 44°) are almost identical. The difference in the two cases is probably due to differences in the temperatures employed in the two sets of experiments and possibly to complications introduced by the effects of other fungi on sweetpotatoes at temperatures near 3.5°. (5) *R. tritici* causes practically all the decay of carrots, whereas *R. nigricans* causes most of the decay of sweetpotatoes, largely because sweetpotatoes as a rule are held at temperatures below 20° at which

which one root in one experiment remained uninfected during the time employed

Although there is some variation in the amount of decay in the different varieties in a given experiment, the variations do not always correspond in the two experiments. Nor is the difference in the amount of decay in the several varieties large enough to indicate marked resistance on the part of any.

The results of the two types of experiments show that all the varieties are very susceptible to infection by *Rhizopus tritici* under the same conditions as those under which Danvers Half Long was found to be readily susceptible, that is, when the roots are inoculated with a spore suspension and stored at a temperature of 30° C. and above, and when they are inoculated by the well method.

#### BACTERIAL SOFT ROT (SLIMY SOFT ROT)

*Bacillus carotovorus* is always present to some extent on vegetables in storage and transit. In addition to decaying carrots in storage and transit, it intermittently occasions losses by attacking the roots in the field. Decay in the field seems to depend on rather special conditions, such as high temperature combined with a high water content of the soil following a severe attack of blight (*Macrosporium carotae* Ell. and Langlois). Although its presence in storage and transit as well as the amount of loss it produces may be influenced by its occurrence in the field, it is not absolutely dependent upon such occurrence.

#### THE PATHOGENE

In the experiments in which the carrot roots were inoculated, the Jones 3A strain of *Bacillus carotovorus* was employed. In the temperature and infection experiments in which no inoculum was used, it is assumed that all the bacterial decay that occurred, except at temperatures of 38° to 40° C. and above, was due to *B. carotovorus*.

#### METHOD OF INFECTION

According to Jones (6), infection of carrots by *Bacillus carotovorus* does not normally occur through the unbroken skin. Unfortunately the skin is not continuous over the entire surface of the roots. Its continuity is interrupted by the wounds produced by harvesting operations, including injury to root tips and secondary roots. These wounds permit of a certain amount of infection whenever conditions of temperature and moisture are favorable (Tables 14 and 15.)

TABLE 14—Normal infection by *Bacillus carotovorus* of wounded and unwounded carrots stored at various temperatures for 31 days

Temperature (° C.)	Wounded roots			Unwounded roots		
	Total		Infected	Total		Infected
	Number	Number		Number	Number	Per cent
24.....	64	58	91	75	50	67
22 5.....	71	18	25	81	18	22
19 5.....	66	20	30	75	10	13
17 5.....	73	5	7	76	2	3
15.....	78	0	0	78	0	0
Total.....	274	101	37	307	80	26

\* Taken from results at temperature at which infection occurred.

## SUSCEPTIBILITY TO RHIZOPUS TRITICI

In two experiments conducted to determine the susceptibility of 16 varieties of carrots to infection and decay by *Rhizopus tritici*, the roots were inoculated by dipping them in a spore suspension, after which they were stored at a temperature of 30° C. The percentage of infection in most varieties was larger in the second experiment (Table 13) than in the first in spite of a shorter storage period. This difference was probably due in part to the heavier spore suspension used in the second experiment. All 16 varieties were found to be susceptible. There was considerable variation in the percentage of infection in the different varieties in a given experiment, but the variation was not always parallel to that of the other experiment. Moreover, the degree of variation in a given variety in the two experiments was sometimes as great as the variation in the different varieties in the same experiment. It is believed, therefore, that there is not a marked difference in the susceptibility of the varieties.

TABLE 13—Infection in 16 varieties of carrots inoculated by dipping in a spore suspension of *Rhizopus tritici* and stored at 30° C.

Variety	Experiment 1					Experiment 2				
	Total roots	Roots infected after—				Total roots	Roots infected after—			
		7 days		15 days			3 days		8 days	
		Number	Number	Per cent	Number		Per cent	Number	Number	Per cent
Blanche à collet vert (hors terre).....	32	6	19	16	50	21	15	71	16	76
Blanche lisse demi-longue.....	30	1	3	21	70	22	14	64	19	86
Carter Early Market.....	70	10	14	34	49	58	10	17	30	52
Carter Nantes.....	70	9	13	48	69	44	26	59	37	84
Carter Red Elephant.....	51	8	16	24	47	47	21	45	36	77
Carter Scarlet Perfection.....	48	2	4	17	35	30	13	43	22	73
Carter Summer Favorite.....	42	13	31	37	88	23	14	61	20	87
Danvers Half Long.....	61	7	11	36	59	48	17	35	40	83
Rouge demi-longue de Danvers.....	56	25	45	46	82	33	13	39	32	97
Jaune obtuse du Doubs.....	38	4	11	21	55	33	17	52	19	58
Rouge à force Parisienne.....	86	53	62	84	98	29	7	24	22	76
Rouge demi-courte de Guérande.....	48	13	27	45	94	32	14	44	30	94
Rouge demi-longue d'Amsterdam.....						41	14	34	38	93
Rouge demi-longue de Chantenay.....	57	10	18	36	63	33	19	58	27	82
Rouge demi-longue Nantaise.....	63	11	17	36	57	30	7	23	25	83
Rouge longue de Saint Valéry.....	55	13	24	29	53	40	21	53	26	65

In two other experiments with these same varieties the roots, after being washed in soap and water, rinsed in tap water, and dried in the laboratory, were inoculated by introducing a 48-hour culture of *Rhizopus tritici* into wells. The wells were made in the thickest diameter of the root, penetrated to the center, and were plugged with cotton. Twenty roots of each variety were employed in each experiment. All the varieties were stored in wire baskets under the same conditions in a room 8 feet square and 10 feet high, provided with ventilation at a temperature of about 23° C. After two days in the first experiment and three days in the second, the roots of each variety were weighed separately, the decay removed, and the undecayed portion weighed again. The weight of the decay was obtained by subtracting the second weight from the first.

The percentage of infection in both experiments was 100, except in the case of the variety Rouge demi-longue de Chantenay, in

tamination by decaying roots on infection and decay at various temperatures, and type 3, to measure the influence of temperature on the quantity of decay at different temperatures.

TYPE 1 —The behavior of carrots stored at different temperatures in regard to infection by *Bacillus carotovorus* within any given period of time is not always uniform either in the range of temperatures at which infection occurs or in the amount of decay present. Aside from the effects of temperature and wounding, the extent of decay is probably influenced by the following factors: The particular lot of carrots stored, the amount of contamination, the humidity of the storage room, and decay by other pathogenes.

The difference in behavior of different lots of carrots and the effects of decay by other pathogenes on decay by *Bacillus carotovorus* will become obvious as the discussion continues. It is a matter of general observation that roots contaminated because of field infection are more likely to decay than roots from uncontaminated fields. A high humidity in the storage room seems to favor infection, although there are no data available to show its exact relation to infection.

The results given in Tables 15 and 16 are submitted as examples of what may happen in regard to infection by *Bacillus carotovorus* if roots are stored at a range of temperatures from 0°–2° to 41° C. In experiment 1 (Table 15) the roots were placed at the various temperatures after having been stored at a temperature of 0° to 2° from November 1 to March 4, while those in experiment 2 were stored at the various temperatures at harvest time. Aside from the difference in the age of the roots at the time of storage, the conditions of the two experiments are not entirely parallel. There are differences in temperature, humidity, and time. The differences in temperature are not believed to be of any importance. The low humidities at the temperatures of 32° and 35° in experiment 1 of Table 15 may possibly operate to reduce the percentage of infection. The temperatures and relative humidities between 15° and 25° employed in the two experiments are sufficiently alike to be fairly comparable. At these temperatures there is a difference in time of three days between experiment 1 (Table 15) and the 19-day period in Table 16. At these temperatures the percentage of infection in newly dug carrots in 19 days (Table 16) was in most instances about the same as in the older roots in 22 days, and at 24° the newly dug carrots showed a much larger percentage of infection than was shown by the older roots at the nearest corresponding temperature (Table 15, experiment 1). These data indicate at least that the roots do not necessarily increase in susceptibility with age. The lower limits for infection in the periods of time considered were about the same. The percentage of infection over the entire infection range in the two experiments was undoubtedly influenced by infection by *Rhizopus* and *Fusarium*, especially the former. Infection by any one of these organisms always shows some variation in amount. Consequently any variation in the percentage of infection by one would tend to cause a variation in the others.

TABLE 15—Influence of temperature and humidity on infection of uninoculated carrots by *Bacillus carotovorus*

Experiment 1 <sup>a</sup>						Experiment 2 <sup>b</sup>					
Tem- perature (° C )	Rela- tive hu- midity	Stor- age period	Roots used	Roots infected		Tem- perature (° C )	Rela- tive hu- midity	Stor- age period	Roots used	Roots infected	
	Per cent	Days	Number	Number	Per cent		Per cent	Days	Number	Number	Per cent
41.....	92	3	86	0	0	36.5	93	12	43	3	7
35.....	57	11	78	3	4	31	91	19	48	12	25
32.....	78	13	60	1	2	24.5	92	27	48	32	67
24.5.....	96	22	70	41	59	21	92	19	57	54	95
22.5.....	93	22	58	14	24	21.5	95	27	59	25	42
20.....	97	22	81	13	16	19	92	27	50	18	36
18.....	96	22	69	10	14	15.5	91	51	63	11	17
14.5.....	95	22	84	0	0	12.5	95	51	48	7	15
11.5.....	95	22	65	0	0	8	91	79	47	0	0
10.....	98	31	56	0	0	6.5	95	113	45	0	0
8.....	90	31	74	0	0	2	94	113	45	0	0
5.....	100	31	66	0	0	1	91	113	61	0	0
3.....	100	31	71	0	0						

<sup>a</sup> Roots exposed to various temperatures after being stored at a temperature of 0° to 2° C, from Nov 1 to Mar 4

<sup>b</sup> Newly dug roots exposed to various temperatures

#### WOUNDING AND INFECTION

Normally the range of temperature extending from 15° to 25° C, and particularly temperatures above 20°, are favorable to infection of carrot by *Bacillus carotovorus*. A quantity of unwounded roots and roots wounded by being struck three times against the blunt edge of a wire basket were stored without inoculation for 31 days at a number of temperatures from 15° to 24°, inclusive. The wounded roots showed a higher percentage of infection than the unwounded roots at all temperatures at which infection occurred. (Table 14.) These results indicate that, although wounding influences infection, heavy infection may take place in the absence of fresh wounding.

#### TEMPERATURE RELATIONS

##### GROWTH OF THE PATHOGENE

Jones (6) found the maximum temperature for growth of *Bacillus carotovorus* on culture media to be slightly below 39° C. Very little growth took place in 20 days at temperatures ranging from 0.6° to 1°. Some growth took place at 2° in 24 hours. The optimum temperature for growth was reported to be approximately 27° to 30°.

#### EXPERIMENTS ON INFECTION AND DECAY

Three types of experiments were employed in the study of the relation of temperature to the infection and development of decay by *Bacillus carotovorus*. In type 1, sound untreated roots were stored at a series of temperatures in infection chambers and storage rooms, in type 2, a root decaying with *B. carotovorus* was placed at or near the center of a quantity of sound roots and the roots were stored at various temperatures; in type 3, the roots were inoculated by the well method and stored at various temperatures. The objects of the three types of experiments were as follows. Type 1, to determine the normal temperature range for infection and decay and the time required for infection to occur, type 2, to study the effects of con-

become high if the duration of the storage period is long enough. With the rise in temperature from 8° to about 21.5°, there is a shortening of the time required for infection to occur. (Table 15, experiment 2, and Table 16.) At temperatures between 21.5° and 36.5° no difference is apparent in the time required for infection to take place, unless a slightly higher percentage of infection at 31° (Table 16) indicates slightly earlier infection. There is very little divergence in the number of infections occurring at these temperatures during the first 8 days. The optimum temperatures for infection among those used (Table 15) were 24° and 24.5°. It is to be expected that the optimum temperature for infection will vary if different temperatures are employed.

TYPE 2.—When bacterial soft rot is present in the field, decaying roots may sometimes be stored with healthy stock and shipped with them to terminal markets, where the usual storage temperatures range from 0° to 2° C. The results in Table 18 were obtained from an experiment designed to throw some light on such cases, as well as on what would happen if such contaminated stock were stored at different temperatures.

In this experiment, eleven 12-quart baskets of sound carrots were inoculated by placing a decaying root in the center of each. The baskets were then stored at temperatures ranging from 2° to 31.5° C. and at relative humidities ranging from 80 to 98 per cent. The rot was the result of inoculation with a pure culture of the Jones 3A strain of *Bacillus carotovorus*. This experiment has some limitations in so far as measuring the influence of temperature on the amount of decay is concerned. Among these are variation in the length of the storage period, variation in the size, shape, and packing of the roots about the inoculum, and the fact that there was only one inoculation at each temperature.

It was desired to obtain infection over the widest possible range of temperature. In attempting to extend infection to as low a temperature as possible, complications developed at the three highest temperatures where the roots showed considerable decay from *Rhizopus* by the end of the twenty-second day.

TABLE 18.—*Influence of temperature on infection of carrots inoculated by placing a root decaying with Bacillus carotovorus in the center of each hamper*

Temperature (°C)	Relative humid- ity	Storage period	Roots used			Roots in- fected			Temperature (°C)	Relative humid- ity	Storage period	Roots used			Roots in- fected		
	Per cent	Days	Num- ber	Num- ber	Per cent					Per cent	Days	Num- ber	Num- ber	Per cent			
31.5.....	90	22	31	2	6	15.5.....			95	29	57	11		19			
30.....	86	22	36	4	11	12.....			90	29	70	7		10			
24.5.....	88	22	45	9	20	8.....			93	29	49	0		0			
23.5.....	91	29	59	12	20	8.....			93	80	49	7		14			
21.....	96	29	36	21	58	5.5.....			98	80	46	3		7			
19.....	80	29	67	15	22	2.....			95	80	45	1		2			

Obviously, the normal variation in size and shape of the carrots and in the packing of them in the baskets would give rise to some variation in the number of roots in a given area about the inoculum. There is always some variation in the amount of decay resulting from a single inoculation. Although the effect of the difference in tempera-

TABLE 16—*Influence of time on infection of uninoculated carrots by Bacillus carotovorus at various temperatures*

Temperature (°C)	Relative humid- ity	Roots used	Roots infected after—							
			4 days	5 days	6 days	8 days	12 days	19 days	27 days	51 days
	Per cent	Number	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
36.5.....	93	43	0	2	5	7	7	-----	-----	-----
31.....	91	48	0	6	6	6	21	25	-----	-----
24.5.....	93	48	0	2	2	2	17	54	67	-----
24.....	95	57	-----	-----	-----	-----	54	95	-----	-----
21.5.....	95	59	0	2	2	2	8	15	42	-----
19.....	92	50	0	0	0	0	6	14	36	-----
15.5.....	91	63	0	0	0	0	0	0	5	17
12.5.....	95	48	0	0	0	0	0	0	0	15

Uninoculated carrots have been infected by *Bacillus carotovorus* at temperatures from 0°-2° to 36.5° C (Table 15, experiment 2, and Table 17)<sup>9</sup> In only one year has bacterial soft rot been observed at a temperature of 0° to 2° during nine years of storage of the Danvers Half Long variety taken directly from the field After 131 days of storage the percentage of infection was 0.2 in this variety but reached as high as 8 in one other variety

TABLE 17.—*Normal infection by Bacillus carotovorus of the Danvers Half Long variety of carrots stored at several temperatures and humidities during four seasons*

Storage season	Temper- ature	Relative humidity	Storage period	Roots used	Roots infected	
	°C	Per cent	Days	Number	Number	Per cent
1924-25.....	10	80-96	102	1,288	80	6
	7	80-96	102	1,190	9	0.8
	4.5	75-83	102	1,029	0	0
	0-2	91	102	1,412	0	0
	10	75-85	121	437	58	13
1925-26.....	7	75-85	121	411	3	0.7
	0-2	90	121	886	0	0
	15.5	90	39	66	22	33
	10	80	99	58	11	19
	4.5	70	165	52	0	0
1926-27.....	0-2	90	131	192	0	0
	0-2	90	131	978	2	0.2
	12.5	91	50	121	53	41
	12.5	91	70	267	205	77
	4.5	84	50	134	0	0
1927-28.....	4.5	84	100	133	5	4
	4.5	84	150	124	0	0
	0-2	90	50	135	0	0
	0-2	90	100	129	0	0
	0-2	90	150	136	0	0
	0-2	86	50	131	0	0
	0-2	86	100	125	0	0
	0-2	86	150	134	0	0
	0-2	86	150	134	0	0
	0-2	86	150	134	0	0

Likewise, infection at 4.5° C was limited to one season out of three seasons' storage of the Danvers Half Long variety (Table 17), the percentage of infection being 4 after 100 days of storage In another season 1 per cent of each of four other varieties was infected after 92 days of storage at 4.5°. Infection at temperatures below 10° has always been relatively small even after long periods of storage. At a temperature of 10° and above, the percentage of infection may

<sup>9</sup> The results recorded in Table 17 were obtained from the storage of carrots at temperatures from 0°-2° to 15.5° during four seasons The roots were taken directly from the field and stored either in 16-quart ampers or bushel crates in storage rooms 8 by 14 by 11 feet high

TABLE 19—*Influence of temperature on infection and decay of carrots inoculated by the well method with Bacillus carotovorus*

[10 roots used in all cases]

Temperature (° C )	Storage period	Roots infected		Decay	Temperature (° C )	Storage period	Roots infected		Decay
	Days	Number	Per cent	Grams		Days	Number	Per cent	Grams
35.....	3	0	0	0	15.5.....	3	9	90	21
32.....	3	0	0	0	12.....	3	5	50	5
29.....	3	2	20	9	8.....	3	0	0	0
26.5.....	3	6	60	42	8.....	7	5	50	1
24.5.....	3	8	80	50	5.5.....	7	1	10	0
21.5.....	3	10	100	41	2.....	12	0	0	0
18.5.....	3	10	100	34					

\* Decay not measurable

The greatest amount of decay occurred at 24.5° C (Table 19, see also Table 15). The amount of decay declined as the temperature rose above or fell below 24.5°. This optimum is slightly below that (27° to 30°) for growth on culture media. The lowest temperature at which infection was obtained in this experiment was 5.5°. Time was undoubtedly a limiting factor.

The temperature at which infection of carrots by *Bacillus carotovorus* occurred in the three types of experiments ranged from 0°–2° to 36.5° C. The optimum temperature for infection is about 25°.

#### INFLUENCE OF RELATIVE HUMIDITY ON INFECTION AND DECAY

Only one experiment was conducted to determine the relation of humidity to infection of carrots by *Bacillus carotovorus*. Unfortunately the temperature used (6.5° C) is normally a limiting factor in infection by this pathogene, and hence the experiment throws little light on the effect of humidity on infection.

Eight 16-quart hampers of freshly harvested, sound carrots were inoculated by dipping them in a suspension of *Bacillus carotovorus* in beef bouillon. The carrots in four of the hampers were dried and one hamper was stored immediately at each of the relative humidities 95, 90, 80, and 70 per cent and at a temperature of 6.5° C. The four other hampers were first stored for 48 hours, without drying, at a temperature of 10° and a relative humidity of 95 per cent, and then placed, one at each humidity, together with the hampers of dried carrots. No infection occurred in the hampers of dried carrots even after 104 days of storage. In a wet hamper first exposed to a temperature of 10° and a relative humidity of 95 per cent for 48 hours and then stored at 6.5° and a relative humidity of 95 per cent, one root was found to be infected after 104 days of storage. Infection was also found on roots that had been given the same preliminary treatment and stored at a relative humidity of 70 per cent. No infection occurred in the wet roots at relative humidities of 80 and 90 per cent. These results are inconclusive as regards the effect of humidity on infection of carrots by *B. carotovorus* except to indicate, perhaps, that the preliminary wetting had some effect. Observations indicate that a high humidity favors infection at the temperatures at which infection normally occurs.

ture tends to overshadow this variation, it does not always do so, and some effects of the initial variation always persist (8) Numbers alone will compensate for this variation.

Infection (Table 18) occurred over the entire temperature range. At 8° C there was no infection in 29 days, but in 80 days there was 14 per cent. The maximum number of infections took place at 21°. This temperature is 3.5° below the optimum obtained in experiments 1 and 2, Table 16. It is believed that 24.5° more nearly represents the optimum for infection and decay. This opinion is confirmed by the results discussed under experiments of type 3, where more quantitative methods were employed. Infection at 8°, 5.5°, and 2° occurred rather slowly. Only 7 roots at 8°, 3 at 5.5°, and 1 at 2° became infected in 80 days. However, in contrast to the results obtained from uninoculated roots, infection occurred at these temperatures in a shorter period of time, in larger amounts, and apparently with greater certainty. At some of the higher temperatures the infection in the same period of time was heavier in uninoculated roots (Table 16) than in the inoculated roots of the present experiment. It is not clear why this should be so, unless the normal contamination aside from the root used for inoculation was less in the latter case than in the case of the uninoculated roots. (Compare results in Table 16 with those in Table 18.) These results show that the inclusion of decaying carrots in stored lots may be a source of decay, the amount depending on the quantity of decaying material present, the temperature of the storage room, and the length of the storage period. This method of inoculation insures infection over a wider temperature range, particularly at low temperatures.

TYPE 3.—For this experiment 180 sound roots of the Danvers Half Long variety were selected for uniformity of shape and size. The results of the test are recorded in Table 19. The roots were first thoroughly washed in soapy water and dried. One hundred and twenty were then inoculated by introducing 0.5 cc of a suspension of *Bacillus carotovorus* in beef bouillon into a well, 8 mm in diameter, penetrating to the center of each root. The wells were plugged with cotton and 10 roots were stored at each of the 12 temperatures shown in Table 19. In 60 roots used as checks sterile beef bouillon, instead of the bacterial suspension, was placed in the wells. Five of these roots were placed at each of the 12 temperatures. The amount of decay was determined by weighing the decaying roots, removing the decay, weighing the undecayed portion, and subtracting the second weight from the first. There was no infection in any of the checks. The highest temperature at which infection occurred in three days was 29° C. This temperature is regarded as the maximum at which decay will take place only for the time limit and other conditions of this experiment. Infection has been obtained at 36.5° (Table 15, experiment 2), but no infection occurred at 41° (Table 15, experiment 1). Thirty-five degrees is 4° below the maximum temperature obtained by Jones (6) for growth of *B. carotovorus* on culture media. The maximum temperature for the infection of carrots by *B. carotovorus* is difficult to determine because of infection by *Rhizopus*, which is always heavy at temperatures above 30°, even within the limits of a few days.

## FACTORS INFLUENCING INITIATION OF INFECTION

## EFFECT OF A NUTRIENT MEDIUM

Some difficulty was experienced in obtaining uniform infection of carrots with *Botrytis cinerea* by artificial inoculation. Uniform infection was desired in order to measure quantitatively the effect of temperature on infection and decay. Inoculation by dipping the roots in a water spore suspension yielded as erratic results as did the storage of carrots at the various temperatures without inoculation. Infection resulting from inserting mycelium and spores into the roots by means of a scalpel was very limited and uncertain. Fairly uniform infection was obtained when the roots were inoculated in wells with a spore suspension in carrot decoction. Less infection occurred when roots were inoculated with a spore suspension in distilled water than when they were inoculated with a spore suspension in carrot decoction (Table 20). These results indicate that the presence of nutrient material other than the carrot tissue in the roots aids infection. Normal infection of carrots by *B. cinerea* in storage seems to be largely associated with and to follow the collapse of certain tissues. The points at which the pathogene gains entrance to the root are the fine tip end of the taproot, secondary roots (rarely), the crown, and wounds. Most of the infection takes place through the fine tip end of the taproot. Very little infection occurs during the first month or two at temperatures from 0° to 4.5° C. During this time some of the fine tip ends, the secondary roots, and some of the top tissues collapse and become necrotic, furnishing weakened or necrotic tissue upon which the pathogene can grow before establishing active relations with the host.

## EFFECT OF WOUNDING

Aside from gaining entrance through the fine tip ends and the tops, *Botrytis* infection takes place almost exclusively through other wounds, especially through fresh wounds, rarely, if ever, through the uninjured skin; and seldom through secondary roots. The results recorded in Table 20 show that no infection occurred through rootlets or through uninjured skin when a spore suspension, either in carrot decoction or in distilled water, was used. Of the roots inoculated with a spore suspension in carrot decoction over fresh wounds, 75 per cent became infected; of those inoculated with a spore suspension in carrot decoction over old wounds, 27 per cent became infected; of those inoculated with a spore suspension in distilled water over fresh wounds, 40 per cent became infected; and of those inoculated with a spore suspension in distilled water over old wounds, none became infected.

In the absence of inoculation, severely wounded roots showed a slightly higher percentage (4 as compared with 0.8) of infection than unwounded roots (Table 21) when stored at the range of temperatures over which *Botrytis* commonly infects carrots. In this case one lot of unwounded carrots and one lot of roots that had been wounded by striking each one on the blunt edge of a wire basket were stored for 31 days at temperatures ranging from 2° to 15° C. The results (Tables 20 and 21) show that the uninjured skin is a barrier to infection and emphasize again the importance of careful handling of carrots in order to avoid wounding and consequent infection.

## VARIETAL SUSCEPTIBILITY TO INFECTION AND DECAY

Three types of experiments were employed in an attempt to measure the relative varietal susceptibility (1) The roots were inoculated by the well method, one-half cubic centimeter of a suspension of *Bacillus carotovorus* in beef bouillon being used for each root. These roots were then stored at 20° C. The quantity of decay was determined by weight as previously described. (2) The roots in a 12-quart hamper of carrots were inoculated by placing at the center a root decaying with *B. carotovorus* and then storing them at 15°. (3) A 16-quart hamper of each variety was stored at harvest time at each of four temperatures, 0°–2°, 4–5°, 10°, and 15–5°, without treatment. Experiments of types 1 and 2 were repeated. In the first type of experiment the criteria used for the measurement of decay were the percentage of infection and the quantity of decay, in the second and third types the criteria used were the number and percentage of carrots infected.

The following 17 varieties of carrots have been found to be susceptible to decay by *Bacillus carotovorus*: Blanche à collet vert (hors terre), Blanche lisse demi-longue, Carter Early Market, Carter Long Forcing, Carter Nantes, Carter Red Elephant, Carter Scarlet Perfection, Carter Summer Favorite, Danvers Half Long, Rouge demi-longue de Danvers, Jaune obtuse du Doubs, Rouge à forcer Parisienne, Rouge demi-courte de Guérande, Rouge demi-longue d'Amsterdam, Rouge demi-longue de Chantenay, Rouge demi-longue Nantaise, and Rouge longue de Saint Valéry.

Although there was considerable variation in infection and decay in the different varieties in a given experiment, the same variation was not maintained in others. If any variety showed greater susceptibility in contrast to other varieties, it was Blanche lisse demi-longue, and even in this case exceptions were found.

## BOTRYTIS ROT OF CARROT (GRAY-MOLD ROT)

## OCCURRENCE

The attention given the Botrytis rot of carrot in literature is confined to a few reports of its occurrence in the field and in storage. It is common in storage, and it always occasions some loss whenever the roots are stored from two to three months at the usual temperatures (0° to 4–5° C.)—the longer the storage period, the greater the loss. As a rule the loss is not large, except in occasional instances after several months of storage.

## THE PATHOGENE

The Botrytis causing decay of carrot in transit and storage is of the *cinerea* type.<sup>10</sup> *Botrytis cinerea*, like *Bacillus carotovorus*, *Rhizopus tritici*, and *R. nigricans*, is nearly ubiquitous and is to some degree omnivorous. It is always present in carrot storage houses and will infect carrots at temperatures ranging from 0° to 7° C. and at relative humidities above 85 per cent, if the period of storage is long enough.

<sup>10</sup> The strain employed in the inoculation experiments was obtained from carrots and identified by Prof. H. H. Whetzel of Cornell University.

## EXPERIMENTS ON INFECTION AND DECAY

Two types of experiments were employed in the study of the relation of temperature to infection and decay. Type 1 consisted of merely storing sound roots at various temperatures for the purpose of observing the infection that normally occurs. Type 2 consisted of a quantitative measurement of decay at various temperatures.

TYPE 1.—The data in Table 23 illustrate (1) the behavior of carrots stored at various temperatures at harvest time, and (2) the behavior of carrots stored at similar temperatures after previous storage at a temperature of about 0° C. In experiment 1, roots were taken directly from the field and stored at temperatures ranging from 1° to 31°. The periods of storage employed correspond very closely to the length of life of the roots. In experiment 2, the carrots were held in storage at a temperature of 0° to 2° from the first week in November until March 4, when they were stored at the various temperatures after the infected roots had been sorted out.

TABLE 22—*Influence of temperature on the growth of Botrytis cinerea on carrot agar in Erlenmeyer flasks*

Temperature (° C)	Average area of colonies after—				Temperature (° C)	Average area of colonies after—			
	3 days	6 days	10 days	12 days		3 days	6 days	10 days	12 days
	Mm <sup>2</sup>	Mm <sup>2</sup>	Mm <sup>2</sup>	Mm <sup>2</sup>		Mm <sup>2</sup>	Mm <sup>2</sup>	Mm <sup>2</sup>	Mm <sup>2</sup>
35.....	0	0	—	—	17.5.....	1,188	—	—	—
32.....	14	—	—	—	13.....	204	3,056	—	—
27.5.....	1,393	—	—	—	11.....	133	2,333	—	—
24.....	1,992	—	—	—	7.....	a 2	127	1,267	—
23.5.....	2,608	—	—	—	6.....	0	102	870	—
18.5.....	1,498	—	—	—	4.5.....	0	18	359	—
					0-1.5.....	0	0	0	182

<sup>a</sup> Just started

TABLE 23—*Influence of temperature on infection of uninoculated carrots by Botrytis cinerea*

Experiment 1 <sup>a</sup>						Experiment 2 <sup>b</sup>					
Temperature (° C)	Relative hu- midity	Storage period	Roots used	Roots infected		Temperature (° C)	Relative hu- midity	Storage period	Roots used	Roots infected	
				Number	Per cent					Number	Per cent
31.....	91	19	48	0	0	32.....	78	13	60	0	0
24.5.....	93	27	48	0	0	24.5.....	96	22	70	13	19
24.....	95	19	57	0	0	20.....	97	22	81	11	14
21.5.....	95	27	59	0	0	18.....	96	22	69	5	7
19.....	92	27	50	0	0	14.5.....	95	22	84	17	20
15.5.....	91	51	63	0	0	11.5.....	95	22	65	11	17
12.5.....	95	51	48	0	0	10.....	98	31	56	7	13
8.....	91	79	47	26	55	8.....	90	31	74	5	7
6.5.....	95	113	45	20	44	5.....	100	31	66	3	5
2.....	94	113	45	22	49	3.....	100	31	71	0	0
1.....	91	113	61	26	43						

<sup>a</sup> Newly dug roots exposed to various temperatures

<sup>b</sup> Roots exposed to various temperatures after being stored at a temperature of 0° to 2° from the first week in November until Mar 4

The results in Table 23, experiment 1, represent what may be expected to happen normally if carrots are stored at various temperatures at harvest time, although infection often occurs at somewhat

TABLE 20—Influence of wounds and of a nutrient medium on infection of carrots inoculated with *Botrytis cinerea* <sup>a</sup>

Temperature (° C )	Storage period	Infection of roots inoculated with spore suspension in—													
		Carrot decoction over—								Distilled water over—					
		Uninjured skin		Fresh wounds		Old wounds		Rootlets		Uninjured skin		Fresh wounds		Old wounds	
		Total	Infected	Total	Infected	Total	Infected	Total	Infected	Total	Infected	Total	Infected	Total	Infected
8	Days 21	No 5	No 0	No 5	No 5	No 5	No 3	No 5	No 0	No 5	No 0	No 5	No 2	No 5	No 0
8	20	5	0	5	4	5	3	5	0	5	0	5	2	5	0
12	25	15	0	6	4	6	0	7	0	5	0	5	2	5	0
Total		25	0	16	12	11	3	12	0	5	0	5	2	5	0

<sup>a</sup> The roots were inoculated by introducing a spore suspension into a glass tube 5 mm in diameter and 8 mm long, sealed over the infection point with vaseline. The fresh wounds were made by removing a small slice of tissue from one side of the root with a knife.

TABLE 21—Normal infection by *Botrytis cinerea* of wounded and unwounded carrots stored at various temperatures for 31 days

Temperature (°C)	Wounded roots			Unwounded roots		
	Total	Infected		Total	Infected	
	Number	Number	Per cent	Number	Number	Per cent
15	78	7	9	78	2	3
11.5	69	2	3	82	1	1
9.5	62	2	3	91	0	0
8	74	3	4	91	1	1
5	65	0	0	96	0	0
2	54	2	4	65	0	0
Total	402	16	4	503	4	1

## TEMPERATURE RELATIONS

## GROWTH OF THE PATHOGENE

To determine the influence of temperature on the growth of *Botrytis cinerea* ten 200-cc Erlenmeyer flasks, each containing 44 cc of carrot agar, were placed at each of 13 temperatures after each flask had been inoculated by introducing a platinum loop of a spore suspension of *B. cinerea* in sterile water into the agar at the center of the flask. No growth occurred at 35° C in six days (Table 22). The highest temperature at which growth occurred was 32°, the maximum amount of growth occurred at 23.5°. As the temperature rose or fell below 23.5°, there was a rapid decline in the rate of growth. Some growth occurred at the lowest temperature employed, 0° to 1.5°. Growth has been observed in another experiment at a temperature of 0.5° to 1°. The cardinal temperatures for growth may be said to be approximately as follows: Maximum, 32° to 35°, optimum, 24°, and minimum, about 0°.

It can not be said on the basis of these results that an increase in temperature within the limits of those given will necessarily result in an increase in the amount of infection by *Botrytis*. In making this statement, due consideration is given the variation in the humidities involved in these experiments, which are not comparable (The storage conditions in these experiments were utilized because they were the best available.) From the results at hand it would seem that humidity was not a limiting factor in these experiments. For instance, at a temperature of 0° to 2° there was very little difference in the amount of infection at 90 and at 86 per cent relative humidity, and contrary to what was expected, infection was greater at the lower humidity. There is often considerable variation in the amount of infection in individual containers stored under the same conditions. To illustrate, the percentage of infection in an individual crate stored at 0° to 2° during the season of 1926-27 was 53, while the average in several crates was 17. The factor involved in this variation is not known. The results obtained during the season of 1927-28 show the relation of time to infection at temperatures of 0° to 2° and 4.5°. Very little infection occurred during the first 50 days. The percentage increased more rapidly during the succeeding intervals of 50 days.

The results in Table 25 were obtained in connection with experiment 1, Table 23, and illustrate the effects of various periods of time on infection at 1°, 2°, 6.5°, and 8° C. In 19 days no infection had occurred in roots stored at 6.5° and 8° and none in 27 days at 1° and 2°. There was a progressive increase in the percentage of infection at each temperature with the lapse of time, except that there was only 44 per cent infection at 6.5° after 113 days of storage as compared with 53 per cent in 79 days at the same temperature. Infection by *Penicillium*, which obscured the previous infection, was responsible for the apparent decrease in infection by *Botrytis*.

It should be stated that the percentages of *Botrytis* infection given, especially at temperatures of 0° to 2° C. in the last three tables, are not necessarily translatable into equal values of losses, especially during the shorter periods of storage. Most of the infection takes place through the broken tip ends of the taproots, and any penetration of the thicker portion of the roots is included in the count. In cases where infection has merely begun to decay the thickened portion of the root, practically the entire root is unimpaired for consumption.

TYPE 2.—As has been stated, some difficulty was experienced in obtaining uniform infection of carrots by *Botrytis*. Quantitative data under such circumstances are rather uncertain. The problem was further complicated at the higher temperatures because of contamination by such pathogens as *Rhizopus* and *Bacillus carotovorus*.

The data given in Table 26 were obtained from one experiment and in the main are representative of the results secured in two other experiments.

The carrots were thoroughly washed and inoculated by introducing an equal quantity of a spore suspension of *Botrytis cinerea* in carrot decoction into a well 2 mm in diameter, penetrating to the center of the thickest portion of the root. They were stored in wire baskets, at the various temperatures shown in Table 26, in infection chambers provided with ventilation. The relative humidity was above 90 per cent at each temperature.

higher temperatures and the percentage of infection varies considerably. As a rule, however, infection does not cover as wide a range of temperature in the storage of freshly dug carrots as in roots previously stored at a temperature of about 0° C. Infection in the freshly dug roots occurred only at temperatures of 1°, 2°, 6.5°, and 8°, whereas in the roots stored in March the range of infection extended from 5° to 24.5° and infection occurred in a somewhat shorter period of time. It should be stated, however, that 24.5° is the highest temperature at which infection has ever been obtained in the storage of untreated carrots. Generally, storage late in the season does not yield infection by *Botrytis* at quite so high a temperature.

The amount of infection by *Botrytis* at temperatures of 6° and above is often influenced by infection by other pathogenes.

The data recorded in Table 24 were obtained in connection with storage of carrots during four seasons in refrigerated rooms either 8 by 14 by 12 feet high, or 8 by 8 by 8 feet. The roots were stored at harvest time in bushel crates or in 16-quart hampers.

TABLE 24—Influence of temperature, humidity, and storage period on the normal infection of carrots by *Botrytis cinerea* during four seasons

Storage season	Temperature	Relative humidity	Storage period	Roots used	Roots infected	
	° C	Per cent	Days	Number	Number	Per cent
1924-25.....	10	80-96	102	1,288	84	7
	7	80-96	102	1,190	68	6
	4.5	75-83	102	1,029	60	6
	0-2	91	102	1,412	89	6
	10	75-85	121	437	112	26
1925-26.....	7	75-85	121	411	40	10
	0-2	90	121	886	49	6
	15.5	90	39	66	10	15
	10	80	99	58	2	3
	4.5	70	165	52	15	29
1926-27.....	0-2	90	131	199	105	<sup>a</sup> 53
	0-2	90	131	978	167	<sup>b</sup> 17
	12.5	91	50	121	0	0
	12.5	91	70	267	0	0
	4.5	84	50	134	2	1
1927-28.....	4.5	84	100	133	8	6
	4.5	84	150	124	58	47
	0-2	90	50	135	3	2
	0-2	90	100	129	6	5
	0-2	90	150	138	21	15
	0-2	86	50	132	3	2
	0-2	86	100	125	15	12
	0-2	86	150	134	25	19

<sup>a</sup> Single crate

<sup>b</sup> Average of several crates.

During the season of 1924-25 there was very little difference in the percentage of *Botrytis* rot present on carrots stored at any of the four temperatures employed. In 1925-26 the percentage of *Botrytis* rot increased with the rise in temperature. The variation in the length of the storage periods was such as to make the results at the different temperatures not comparable. At temperatures of 10° and 15.5° C infection by *Botrytis* was complicated by infection by *Bacillus carotovorus* and forms of *Fusarium*. During the season of 1927-28 the percentage of infection after 50 to 100 days of storage was greater at 0° to 2° (if infection at the two humidities at this temperature is considered) than at 4.5°. After 150 days of storage the percentage of infection at 4.5° was 47 as compared with 19 per cent at 0° to 2°. No infection occurred at 12.5°, largely, it is believed, because of heavy infection by *B. carotovorus* and forms of *Fusarium*.

the roots in a spore suspension of the pathogene, the other four hampers (checks) were stored, without treatment, at the relative humidities of 95, 90, 80, and 70 per cent, respectively, and at a temperature of 6.5° C. Four hampers of the inoculated roots were poured out on a clean floor and dried by means of an electric fan. The roots were then returned to the dry hampers. One hamper each of dried and undried (wet) inoculated carrots was stored with each of the checks. The length of the storage period was 104 days.

TABLE 27—*Influence of humidity on the infection of carrots inoculated with Botrytis cinerea and stored for 104 days at a temperature of 6.5° C*

Relative humidity (per cent)	Inoculated roots						Untreated roots		
	Wet			Dried					
	Total	Infected		Total	Infected		Total	Infected	
	Number	Number	Per cent	Number	Number	Per cent	Number	Number	Per cent
95.....	139	9	6	107	8	7	106	10	9
90.....	127	9	7	118	10	8	100	6	6
80.....	126	2	2	108	2	2	132	11	8
70.....	124	2	2	129	0	0	107	0	0

There was no significant difference in the percentage of infection in either the inoculated or uninoculated roots at relative humidities of 90 or 95 per cent. As the relative humidity fell from 90 to 80 per cent there was a considerable drop in the percentage of infection in the inoculated roots, but not in the checks. There was no infection in the dried inoculated roots or checks at a relative humidity of 70 per cent. At this humidity there was only 2 per cent infection in the wet inoculated roots, the same percentage of infection was obtained in wet roots at 80 per cent relative humidity. These results indicate that a relative humidity of 70 per cent and possibly of 80 per cent is unfavorable to infection. This humidity, however, is also unfavorable for the storage of carrots.

#### VARIETAL SUSCEPTIBILITY TO INFECTION AND DECAY

In addition to the 14 varieties of carrot listed in Table 28, the following 4 varieties have been found susceptible to infection and decay by *Botrytis cinerea*. Blanche à collet vert (hors terre), Blanche lisse demi-longue, Jaune obtuse du Doubs, and Carter Summer Favorite.

Two types of experiments were conducted to determine the relative susceptibility of the different varieties. In one type the roots were artificially inoculated, and in the other infection was dependent on the inoculum normally present on the roots.

#### INOCULATION EXPERIMENTS

In each of two experiments, 20 carrots of each variety were washed in soapy water and dried in the laboratory. By means of a hypodermic needle, 0.2 cc of a spore suspension of *Botrytis cinerea* in carrot decoction was then introduced into a well 2 mm in diameter and 2 cm deep in the thickest diameter of the root. The roots were stored

TABLE 25 —Influence of storage period on infection of uninoculated carrots by *Botrytis cinerea* at various temperatures

Temperature (° C )	Relative humidity	Roots used	Roots infected after—			
			27 days	51 days	79 days	113 days
	Per cent	Number	Per cent	Per cent	Per cent	Per cent
8.....	91	47	4	25	55	—
6.5.....	95	45	2	24	53	44
2.....	94	45	0	9	31	40
1.....	91	61	0	8	25	39
1.....	91	* 152-197	—	6	—	25

\* These carrots were stored in crates in a storage room, whereas the remaining lots were stored in hampers in infection chambers. One crate (containing 152 roots) was inspected after 51 days and one (containing 197 roots) was inspected after 113 days.

TABLE 26 —Influence of temperature on infection and decay of carrots inoculated with *Botrytis cinerea* <sup>a</sup>

[5 roots used in all cases]

Temperature (° C )	Storage period	Roots infected		Average area of lesions	Temperature (° C )	Storage period	Roots infected		Average area of lesions
		Number	Per cent				Number	Per cent	
30.....	Days	0	0	0	9.5.....	9	5	100	273
28.....	4	0	0	0	7.....	9	1	20	( <sup>b</sup> )
24.5.....	4	4	80	234	5.....	9	0	0	0
22.5.....	4	5	100	344	7.....	17	5	100	410
20.....	4	5	100	240	5.....	17	5	100	111
18.5.....	4	5	100	240	2.....	17	0	0	0
12.....	4	0	0	0	2.....	42	3	60	474
12.....	9	5	100	654					

<sup>a</sup> The relative humidity of the storage chambers was above 90 per cent.

<sup>b</sup> Just started.

The highest temperature at which infection occurred in this experiment was 24.5° C (Table 26). In another experiment infection was obtained at 28°. Both temperatures are somewhat below the maximum for growth of *Botrytis cinerea* (32°) (Table 22). This might well be expected, since infection by *Botrytis* at the higher temperatures is somewhat uncertain (Table 23, experiment 1). The greatest amount of decay after four days occurred at 22.5°, an optimum temperature corresponding closely with that for growth of this pathogen on culture media. After nine days there was 654 mm<sup>2</sup> decay at 12°, 273 mm<sup>2</sup> at 9.5°, a trace at 7°, and none at 5°. The amount of decay declined during this period as the temperature rose above or fell below 22.5°. Considerable decay occurred at 2° in 42 days in three out of five roots. Infection has occurred at temperatures as low as 0°.

The maximum and minimum temperatures for infection based on all temperature experiments conducted are approximately 24.5° to 28° and 0° C, respectively. The optimum for the rate of decay is about 23°.

#### INFLUENCE OF HUMIDITY ON INFECTION AND DECAY

The data available are too few to permit one to draw definite conclusions regarding the influence of humidity on infection of carrots by *Botrytis cinerea*. In one experiment (Table 27) freshly dug carrots were employed. Eight hampers of carrots were inoculated by dipping

## EXPERIMENTS WITH UNINOCULATED ROOTS

A 16-quart hamper of each of the varieties listed in Table 29 was taken directly from the field (October 28, 1926) and stored at each of four temperatures (0 to 2°, 4.5°, 10°, and 15.5° C) in storage rooms 8 by 14 by 11 feet high. The hampers were placed at the same level in each room. Hampers stored at 0° to 2° were placed between two shelves in a space just high enough for them. A second lot of each variety<sup>11</sup> was stored in bushel crates at 0° to 2° in the same room as the hampers. The crates were stacked five high, in adjacent tiers.

TABLE 29—*Susceptibility of 17 uninoculated varieties of carrot to infection by Botrytis cinerea*

Variety	Percentage of roots infected at indicated temperature (°C), relative humidity (per cent), and storage period (days) when—				
	Stored in 16-quart hampers				Stored in bushel crates (0°-2°, 90 per cent, 131 days)
	15.5°, 90 per cent, 39 days	10°, 80 per cent, 99 days	4.5°, 70 per cent, 165 days	0°-2°, 90 per cent, 131 days	
Blanche à collet vert (hors terre).....	32	0	6.5	35.2	5.8
Blanche lisse demi-longue.....	0	9.5	18.1	51.4	34.4
Carter Early Market.....	11.3	1.7	12.3	25.8	18.8
Carter Long Forcing.....	13.1	0	-----	28.2	-----
Carter Nantes.....	17	0	16.7	27.2	9.5
Carter Red Elephant.....	8.9	0	16.7	22.1	18.8
Carter Scarlet Perfection.....	22.7	2.6	27.7	29.1	6.7
Carter Summer Favorite.....	9.8	2.2	10	14.8	19.6
Danvers Half Long.....	15.2	3.5	28.8	52.8	17.1
Jaune obtuse du Doubs.....	8.3	0	32.1	34.7	14.5
Rouge demi-longue de Danvers.....	13.4	0	7.5	26.9	14.8
Rouge à force Parisienne.....	15.5	4.1	25.6	25.9	25.4
Rouge demi-courte de Guérande.....	2.9	6.2	16.1	21.7	8.2
Rouge demi-longue d'Amsterdam.....	4.2	1.7	10.4	21.5	20.9
Rouge demi-longue de Chantenay.....	27	3	15.5	21.9	15.3
Rouge demi-longue Nantaise.....	5	2.3	10.7	14.2	2.2
Rouge longue de Saint Valéry.....	14.3	4.7	6.8	17.4	1.9

In all varieties, except Carter Summer Favorite, the percentage of infection at 0° to 2° C. was higher in the hamper lot than in the crate lot, and in most varieties considerably higher. Just why there should have been so marked a difference in the two lots is not entirely clear. The relative humidity readings were taken in the open part of the room and represent the humidity of the air surrounding the crates rather than that surrounding the hampers. It is possible, because of the position of the hampers between the shelves and their closeness to each other, that the humidity was higher and that there was less ventilation in the hampers than in the crates. These factors may have increased the amount of infection. In any case it is probable that the difference in the percentage of infection in the two lots was not wholly accidental.

The results at the different temperatures can not be compared directly, desirable though such a comparison would be, because of the differences in the humidity of the storage rooms and in the duration of the storage periods.

The data at 10° and 15.5° C. show considerable variation in the percentage of infection, and in a number of cases a complete absence

<sup>11</sup> Carter Long Forcing was not stored at 4.5° or in crates at 0° to 2°

in wire baskets at the same level in a room 8 by 8 by 8 feet, at a temperature of 12° C. The length of the storage period in the first experiment was 12 days and in the second 16 days.

The criteria used in measuring the relative susceptibility of the several varieties were (1) Percentage of roots infected, (2) average diameter of the lesions based on the total number of inoculations, and (3) average diameter of the lesions that developed in each variety. The diameters of the lesions were obtained from the cross sections of the roots cut in two directly lengthwise through the well. The first two criteria are measures of the ability of the fungus to infect and the amount of decay occasioned, and the third the measure of its ability to penetrate the tissue after it has established relations with the host.

TABLE 28—*Susceptibility of 14 varieties of carrot to decay when inoculated with Botrytis cinerea* \*

Variety	Roots infected			Average diameter of lesions based on—					
				Number of roots			Number of lesions		
	Experiment 1	Experiment 2	Mean	Experiment 1	Experiment 2	Mean	Experiment 1	Experiment 2	Mean
	Per cent	Per cent	Per cent	Mm	Mm	Mm	Mm	Mm	Mm
Carter Early Market.....	95	80	88	21	23.8	22.2	20	19	19.5
Carter Long Forcing.....	33	30	32	15.4	11.5	13.6	5.1	3.5	4.3
Carter Nantes.....	90	90	90	22.1	25	23.5	19.8	22.3	21.7
Carter Red Elephant.....	95	89	92	20.6	22.6	21.5	19.5	20	19.8
Carter Scarlet Perfection.....	52	80	66	17.4	20.4	19.1	9.1	16.4	12.6
Danvers Half Long.....		90	90		21	21		19	19
Rouge demi-longue de Danvers.....	90		90	19.5		19.5	19.5		19.5
Rouge à forcer Parisienne.....	85	90	88	21.8	28.8	25.3	18.5	25.1	21.8
Rouge demi-courte de Guérande.....	100	90	95	22.4	25.5	24	22.4	23	23.9
Rouge demi-longue d'Amsterdam.....	100	84	92	18.9	21.4	20	18.9	18	18.5
Rouge demi-longue de Chantenay.....	85	55	70	21.4	23.4	22.2	18.2	12.9	15.5
Rouge demi-longue de Saint James.....	84		84	17.3		17.3	14.6		14.6
Rouge demi-longue Nantaise.....	100	90	95	19.4	22	20.6	19.4	19.8	19.6
Rouge longue de Saint Valéry.....	80	100	90	22.2	25.8	24	17.8	25.8	21.6

\* Duration of first experiment, 12 days, of second experiment, 16 days.

The percentage of infection in this case is no doubt a more accurate index to susceptibility than the diameter of the lesions because of certain difficulties arising from the size of the roots in the several varieties and the variation in the relative thickness of the core and cortical tissue, which affect the rate of penetration and hence the diameter of the lesions. The cortical tissue, at times at least, seems to be more resistant to decay than the core and tends to limit the degree of decay. Any variation in the amount of cortex and core would therefore affect the amount of decay. The size of the roots would also influence this variation, for the advance of decay would be inhibited earlier in the smaller roots than in the large ones. Only one variety (Table 28), Carter Long Forcing, consistently showed a considerable degree of resistance when judged by all three criteria in both experiments. Two other varieties, Carter Scarlet Perfection and Rouge demi-longue de Chantenay, showed a somewhat lower percentage of infection than the other 15 varieties. Most of the varieties were highly susceptible.

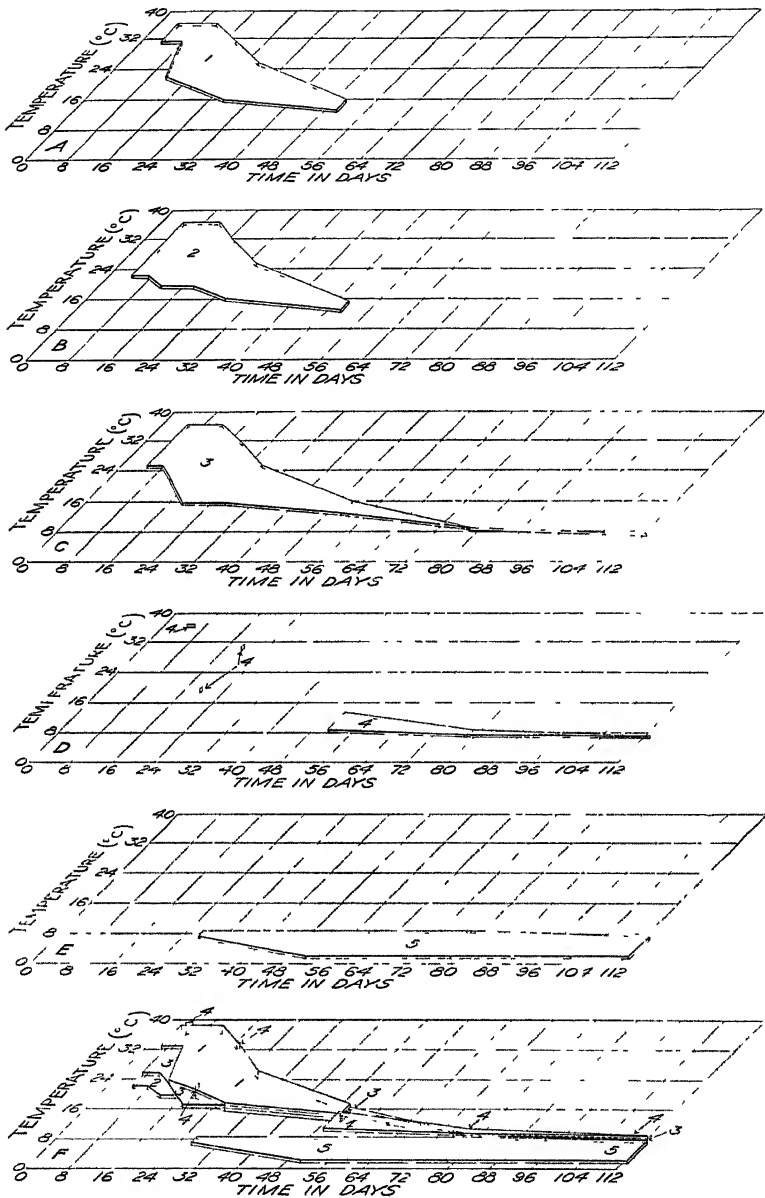


FIGURE 1—Influence of temperature and time on the occurrence of infection by the organisms that normally decay carrots when stored at a range of temperatures extending from 1° to 36.5°  
C A *Rhizopus*, B *Bacillus carotovorus*, C *Fusarium*, D *Penicillium*, E *Botrytis cinerea*,  
of A, B, C, D, and E, combination F, represented by 1, 2, 3, 4, and 5, respectively

of infection. A large part of the variation was due to bacterial and *Penicillium* decay. For instance, in the case of the Blanche lisse demi-longue variety, all the roots stored at 15.5° were decayed by *Bacillus carotororus*. At a temperature of 4.5°, infection by *Penicillium* was rather severe in some cases and may have influenced the amount of *Botrytis* infection.

At temperatures of 0° to 2° and 4.5° C, in both hamper and crate storage, there was considerable variation in the percentage of infection in the different varieties, but the variation in the case of any one variety under the three conditions was not striking or consistent enough to make it possible to draw very definite conclusions as to variation in resistance. Carter Long Forcing, instead of showing the greatest resistance among the varieties with which it is compared in Table 28, shows less resistance than certain other varieties.

As a result of these experiments it may at least be inferred that all the varieties tested are susceptible to infection and decay by *Botrytis* and that the small amount of resistance shown by any one variety does not give it much advantage over the other varieties.

### DISCUSSION

#### TEMPERATURE AS A GOVERNING FACTOR IN THE INFECTION AND DEVELOPMENT OF STORAGE DISEASES OF CARROTS

Aside from such diseases as *Sclerotinia* soft rot and black rot (*Alternaria radicina* Meier, Drechs, and Eddy), which directly or indirectly have their origin in the field, diseases that develop in the storage house are governed largely by temperature, provided that the relative humidity of the storage room is high enough to prevent shriveling.

If carrots are stored at harvest time at temperatures ranging from 1° to 37° C, results similar to those presented in Table 30 are usually obtained. The percentage of infection and the temperature limits of the various diseases may be expected to vary somewhat. It may be added as supplementary to these data that carrots stored at temperatures above 37° often show evidence of injury aside from infection, manifested in the blackening and killing of the tissue. The length of life of the roots at these temperatures is only one or two days, and *Penicillium* invades most of the roots and sometimes all of them. In addition to *Penicillium*, a very sticky bacterium is found particularly associated with the injured roots. The diseases that normally develop in roots stored at temperatures from 0° to 37° are *Rhizopus* soft rot, bacterial soft rot, *Fusarium* rot, *Penicillium* rot, and *Botrytis* rot. Unidentified lesions also develop sometimes, and upon very rare occasions *Rhizoctonia solani* Kuhn infects carrots at temperatures near 0°. Because of the overlapping of the temperature ranges of infection by some of the pathogenes, the amount of decay produced by any one pathogene was influenced by that produced by another.

Figure 1 illustrates the distribution of infection by the various pathogenes at the different temperatures during the different periods of time. A, B, C, D, and E represent the separate distribution of infection by *Rhizopus*, *Bacillus carotororus*, *Fusarium*, *Penicillium*, and *Botrytis cinerea*, respectively, and F is a composite graph of the distribution of infection by the five pathogenes. The graphs are not intended to convey any idea of the quantity of decay present but merely to show the occurrence of infection at the different temperatures.





TABLE 31 —*Influence of temperature on sprouting of carrot roots*

Temperature (°C)	Time re- quired for roots to begin sprouting	Temperature (°C)	Time re- quired for roots to begin sprouting	Temperature (°C)	Time re- quired for roots to begin sprouting	Temperature (°C)	Time re- quired for roots to begin sprouting
	Days ( <sup>a</sup> )		Days		Days		Days ( <sup>b</sup> )
36.5.....	5	21.5.....	5	12.5.....	12	2.....	( <sup>b</sup> )
31.....	5	19.....	5	8.....	19	1.....	( <sup>b</sup> )
24.5.....	5	15.5.....	12	6.5.....	27		

<sup>a</sup> None in 12 days<sup>b</sup> None in 113 daysTABLE 32 —*Development of diseases in the Danvers Half Long variety of carrot under various conditions of storage during four seasons*

Storage season	Temperature	Relative humidity	Storage period	Roots used	Roots infected	Percentage of roots infected with—							Roots infected at tip ends <sup>a</sup>	Sound roots plus roots infected at tip ends
						Bacterial soft rot	Black rot	Fusarium rot	Penicillium rot	Rhizopus rot	Unidentified decay	Botrytis rot		
	°C	Per cent	Days	Num-ber	Per cent								Per cent	Per cent
1924-25	10	80-96	102	1,288	60	6	1	0	0	0	0	7	46	86
	7	80-96	102	1,190	41	0	4	0	0	0	0	6	34	93
	4	75-83	102	1,029	20	0	6	0	0	0	0	6	13	93
	0-2	91	102	1,412	17	0	2	0	0	0	0	6	11	94
1925-26	10	75-85	121	437	50	13	7	0	2	2	4	26	17	58
	7	75-85	121	411	35	7	7	0	4	3	0	10	19	84
	0-2	60	121	886	24	0	1	0	0	0	0	6	14	90
	15.5	90	39	66	68	33	7	33	6	0	0	15	14	16
1926-27	10	80	99	58	61	19	4	0	2	0	0	3	0	36
	4	70	165	52	58	0	0	0	25	0	0	24	0	42
	0-2	90	131	199	48	0	1	0	0	0	0	53	35	47
	0-2	90	131	978	51	2	0	0	9	1	4	17	32	81
1927-28	12.5	91	50	121	81	44	7	21	0	0	16	0	0	19
	12.5	91	70	267	94	77	0	10	0	0	6	0	0	6
	4.5	84	50	134	16	0	0	1	0	0	0	3	12	96
	1.5	81	100	133	28	4	3	1	0	0	0	11	12	84
	4.5	81	150	124	60	0	0	0	0	0	0	47	10	50
	0-2	90	50	135	5	0	2	0	0	0	0	2	3	98
	0-2	90	100	129	20	0	1	0	0	0	0	5	13	93
	0-2	90	150	138	31	0	0	0	0	0	0	15	15	84
	0-2	86	50	132	3	0	0	0	0	0	0	2	1	98
	0-2	86	100	125	22	0	0	0	0	0	0	12	10	88
	0-2	86	150	134	43	0	0	1	0	0	0	19	23	80

<sup>a</sup> The decay under this heading is confined to the thin portion at the root tip and does not affect the market value of the carrot

Fusarium rot was present each season except one, but the amount of loss that it occasioned varied greatly. Although its development is influenced largely by temperature, its presence or absence seems to be governed by some other factor. In one instance (1927-28) infection occurred at a temperature of 0° to 2° C; otherwise infection by Fusarium has never been observed at this temperature and very little has been found at temperatures below 8°.

Penicillium was present during two seasons and occurred at as low a temperature as 0° to 2° C. Its presence or absence at these low temperatures seems to be largely governed by some factor other than temperature.

A large percentage of the carrots were infected each season at the fine tip ends of the roots. The infection so listed in the table never reached the thickened portion of the root and did not materially

during different periods of time. The graphs are incomplete in that they do not show the infection that occurs above  $36.5^{\circ}$  and below  $1^{\circ}$  C. Furthermore, the periods of time given do not always correspond at the lower limits to the time required for the initial infection to occur at a given temperature. The graphs do, however, give some idea of the distribution of infection, the conditions of temperature and time in which infection is absent, and the extreme length of life of the carrots at temperatures above  $6.5^{\circ}$ . The recording of data at these temperatures was discontinued only when the deterioration of the roots was such as to preclude their consumption. The time required for the roots to reach this state of deterioration is approximately represented by the upper receding line of each graph. This line marks the discontinuance of recording data at the various temperatures.

The data presented in Table 30 and Figure 1 show that if carrots are stored at or near  $1^{\circ}$  C the limiting disease originating in storage is *Botrytis* rot. Fortunately its development is very slow. The other diseases become limiting factors only when roots are stored or transported at temperatures unfavorable for the holding of carrots for reasons other than infection. Table 31 shows that sprouting begins in roots held at temperatures of  $19^{\circ}$ ,  $21.5^{\circ}$ ,  $24.5^{\circ}$ , and  $31^{\circ}$  in 5 days. No sprouting occurred at  $36.5^{\circ}$  in 12 days. Sprouting began at  $15.5^{\circ}$  and  $12.5^{\circ}$  in 12 days, at  $8^{\circ}$  in 19 days, and at  $6.5^{\circ}$  in 27 days, whereas at  $1^{\circ}$  and  $2^{\circ}$  no sprouting was apparent in 113 days. It is evident, therefore, that decay by *Rhizopus*, *Bacillus carotovorus*, *Fusarium*, and *Penicillium* occurs, as a rule, only when deterioration from other causes has set in. The sprouts themselves soon deteriorate at temperatures of  $10^{\circ}$  and above, because of infection by bacteria and fungi. As a result of this latter infection, the roots become discolored and slimy.

#### DEVELOPMENT OF DISEASE AT COMMON STORAGE TEMPERATURES

Roots of the Danvers Half Long variety were stored four different years at various ranges of temperatures the extreme limits of which were  $0^{\circ}$  to  $2^{\circ}$  and  $15.5^{\circ}$  C (Table 32.) The range probably covers that usually employed in common storage, and the results illustrate the losses that might be expected provided *Sclerotinia* soft rot was not present.

Bacterial soft rot was consistently present in considerable quantity at temperatures of  $10^{\circ}$  C and above. The losses below  $10^{\circ}$  were very slight, and only in one season, 1926-27, did bacterial soft rot (0.2 per cent) occur at  $0^{\circ}$  to  $2^{\circ}$ .

Some black rot occurred each season, but the amount present had no relation to temperature. Moreover, it was not always present under all the conditions of storage, although it develops readily under all these conditions, indicating that the pathogene is not as ubiquitous as *Bacillus carotovorus* and *Rhizopus*.

The results of the year 1927-28, it is believed, give a fair picture of the possible losses that might be expected after various periods of storage, because the greatest total loss was incurred during this season. It is true that in two of the seasons the storage period was shorter, but in two of them it was longer.

#### VARIETAL SUSCEPTIBILITY TO DECAY AT COMMON STORAGE TEMPERATURES

The results recorded in Table 34 were obtained from one season's storage of 17 varieties of carrots at temperatures of 0° to 2°, 4 5°, 10°, and 15 5° C in 16-quart hampers and at 0° to 2° in crates.

The roots in hampers were stored on the same level at each temperature, whereas those in crates were stored in mass. The results are representative in the main of those obtained during two other seasons. An exception is the development of bacterial soft rot at a temperature of 0° to 2° C; in fact, bacterial soft rot has never been found at any other time at this temperature during nine years of storage of the Danvers Half Long variety.

affect the market value of the carrots, because the diseased tips would usually be broken off in the course of preparation for the market. From the standpoint of infection, roots showing the percentages given in the last column of Table 32 might be regarded as fit for market. Because of germination and infection of the tops and discoloration of the roots, the carrots stored at temperatures above 4 5° C were unfit for market, whereas those stored at 0 2° and 4 5° C are regarded as still unimpaired in market value.

Numerous isolations were made from the infected tip ends. The organisms obtained varied greatly. Among them were *Botrytis cinerea*, *Bacillus carotovorus*, *Alternaria radicina*, and forms of *Fusarium* and *Penicillium*. Because of the small size of these fine tip ends and the fact that the decay involves the entire cross section, one is almost as likely to get a contaminating organism as the pathogene. At temperatures between 0° and 5° C, most of these infections ultimately lead to *Botrytis* decay.

The results for the season 1927-28 (Table 32) illustrate the progress of decay during storage at three temperatures and in the case of 0°-2° C at two humidities. Lower humidities were employed at each temperature, but the roots were so badly shriveled from loss of water that the results are not submitted.

At 12 5° C very few roots remained sound after 50 days of storage. At 4 5° the loss was only 4 per cent in the first 50 days, but by the end of 150 days only 50 per cent remained marketable. At 0° to 2° there was very little difference in the losses at the two humidities, and 80 and 84 per cent of the roots were marketable after 150 days.

TABLE 33—Infection of the Danvers Half Long variety of carrots stored for various periods at 0° to 2° C during five seasons

Storage season	Relative humidity	Storage period	Roots used	Roots infected	Percentage of roots infected with—							Roots infected tip ends *	Roots marketable
					Bacterial rot	Black rot	Fusarium rot	Penicillium rot	Rhizopus soft rot	Unidentified decay	Botrytis rot		
	Per cent	Days	Number	Per cent								Per cent	Per cent
1923-24.....	91	201	1,727	57	0	0 2	0	0	0	0	16	41	84
1924-25.....	91	102	1,412	17	0	2	0	0	0	0	6	11	94
1925-26.....	90	161	886	24	0	4	0	0	0	0	6	14	90
1926-27.....	85	131	978	50	2	1	0	9	1	4	17	31	81
1927-28.....	85	50	131	3	0	0	0	0	0	0	2	2	98
	85	100	125	22	0	0	0	0	0	0	12	10	88
	85	150	134	43	0	0	8	0	0	0	19	23	80

\* The decay in this case is confined to thin portions of root tip and does not affect the marketable value of the carrot.

The results recorded in Table 33 give the percentage of losses due to different diseases of the Danvers Half Long variety during five different years of storage at 0° to 2° C and the progress of decay during the season 1927-28. The duration of the storage ranged from 50 to 201 days and the losses for the season from 2 to 20 per cent. Most of the losses were due to *Botrytis* rot and included roots that showed any infection whatever of their thickened portion.

Variety	Percentage of carrots infected at indicated temperature (° C ) and humidity (per cent)												Percentage of sound roots plus roots infected at tip ends only at indicated temperature (° C ) and humidity (per cent)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
	By unknown cause						At tip ends only						Total c																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
	4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°			0°-2°			15 °5°			10°			4 °5°		

a Of the two columns of data under 0°-2° in each group, the first refers to roots stored in 10-quart hampers, the second to roots stored in crates  
 b The storage periods were At 15°, 39 days, at 10°, 165 days, at 4°, 165 days, and at 0°-2°, 131 days  
 c Totals refer to all preceding data. Small discrepencies are due to the fact that the total infection was not calculated but was recorded from observation  
 d Five per cent were infected with Rhizopus

TABLE 34.—*Influence of certain temperatures in storage on normal infection of 17 varieties of carrots a b*

Variety	Percentage of carrots infected at indicated temperature (° C) and humidity (per cent)																							
	By <i>Bacillus carotovorus</i>						By <i>Fusarium</i>						By <i>Penicillium</i>						By <i>Botrytis cinerea</i>					
	15° 90	10° 80	4° 70	0°-2° 90	0°-2° 90	15° 90	10° 80	4° 70	0°-2° 90	0°-2° 90	15° 90	10° 80	4° 70	0°-2° 90	0°-2° 90	15° 90	10° 80	4° 70	0°-2° 90	0°-2° 90	15° 90	10° 80	4° 70	0°-2° 90
Blanche à collet vert (hors terre).....	32	14	0	1	1	0	0	0	0	0	0	0	4	0	0	32	0	7	35	6	0	3	0	0
Blanche lisse demi-longue.....	100	81	0	0	5	7	0	0	0	0	0	0	23	0	0	0	0	18	51	34	0	0	5	0
Carier Early Market.....	08	22	5	2	7	0	0	0	0	0	0	0	7	0	0	11	2	12	26	19	2	3	15	2
Carier Long Foreing.....	64	64	11	8	11	0	0	0	0	0	0	0	5	0	0	0	0	28	28	0	0	0	0	4
Carier Nantes.....	36	11	0	0	0	0	0	0	0	0	0	0	4	0	0	17	0	17	27	10	0	0	10	1
Carier Red Elephant.....	48	32	0	1	3	0	0	0	0	0	0	0	11	0	0	17	0	17	22	19	0	0	0	6
Carier Scarlet Perfection.....	69	67	0	3	5	0	0	0	0	0	0	0	10	0	0	9	0	28	29	7	0	0	0	0
Carier Summer Favorite.....	48	8	0	0	0	0	0	0	0	0	0	0	15	0	0	10	2	10	15	20	2	0	0	9
Carier Scarlet Perfection.....	33	69	0	3	5	0	0	0	0	0	0	0	23	0	0	13	0	24	33	17	0	7	4	0
Danvers Long.....	29	29	0	2	0	0	0	0	0	0	0	0	25	0	0	18	0	8	27	15	4	0	5	1
Rouge demi-longue de Danvers.....	74	69	0	0	0	0	0	0	0	0	0	0	30	0	0	16	0	32	35	23	2	4	0	0
Rouge obtuse du Doubs.....	44	69	0	0	0	0	0	0	0	0	0	0	17	0	0	16	4	26	26	23	0	3	0	1
Rouge à forcer Parisienne.....	88	59	0	0	9	4	0	0	0	0	0	0	3	0	0	3	0	10	22	23	2	2	7	4
Rouge demi-courte de Guérande.....	67	47	0	0	2	0	0	0	0	0	0	0	10	0	0	1	2	18	22	24	0	3	18	0
Rouge demi-longue d'Amsterdam.....	41	42	0	0	8	1	0	0	0	0	0	0	2	0	0	27	0	3	18	15	0	0	0	2
Rouge demi-longue de Chantenay.....	50	40	0	0	8	0	0	0	0	0	0	0	2	0	0	15	2	11	14	2	0	0	0	0
Rouge demi-longue Nantes.....	53	21	0	2	1	0	0	0	0	0	0	0	34	0	0	14	5	7	17	2	0	2	0	1

TABLE 35—Influence of humidity on shriveling of carrots stored at 6.5° C

Relative humidity (per cent)	Storage period	Roots used	Roots shriveled			Relative humidity (per cent)	Storage period	Roots used	Roots shriveled		
			Number	Number	Per cent				Number	Number	Per cent
95	47	726	0	0	0	95	104	723	0	0	0
90	47	596	0	0	0	90	104	622	0	0	0
80	47	694	116	17	2.5	80	104	220	31	14	14
70	47	710	367	52	7.3	70	104	253	206	81	81

## SUMMARY AND CONCLUSIONS

The market-inspection certificates issued by the United States Department of Agriculture on the 214 cars of topped carrots inspected in the United States during the years 1922 to 1927, inclusive, show the following average percentages of decay: 6.3 per cent from *Sclerotinia* soft rot, 2.6 per cent from *Rhizopus* soft rot, 1.2 per cent from bacterial soft rot, and 2.9 per cent from *Botrytis* rot.

Since a large number of these cars were inspected for condition and grade independent of decay, it is believed that these figures give some indication of the losses and their causes in the carrots inspected and a suggestion of the losses that generally occur in transit.

Although the uninjured skin of carrot roots is an effective barrier against infection by *Sclerotinia sclerotiorum*, yet because of the discontinuity of the skin due to old wounds, secondary roots, and the broken tip ends of the taproots, infection takes place readily whenever the pathogene is present.

Fresh wounding, although not indispensable to infection, increases the amount.

*Sclerotinia sclerotiorum* was found to grow on carrot agar at temperatures ranging from 0.9° to 32.5° C. No observations were made at temperatures below 0.9°. The maximum amount of growth occurred at 24°.

Infection of carrots by *Sclerotinia sclerotiorum* has been obtained at temperatures ranging from 0°–1° to 28° C. The greatest amount of decay was obtained at 23°. The difference between the optimum for growth of *S. sclerotiorum* on agar and that for decay of carrots by the same organism is believed to be due to the difference in the temperatures employed in the two cases. Although it is not practicable to store carrots below the temperature limit for infection by *S. sclerotiorum*, the processes of infection and decay proceed very slowly at or near 0°. Since the *Sclerotinia* soft rot has its origin directly or indirectly in the field, its control should begin in the field.

Roots dipped in a suspension of mycelium of *Sclerotinia sclerotiorum* and agar in water and stored while wet at relative humidities of 80 and 90 per cent at a temperature of 6.5° C. became infected. The percentage of infection was much higher at 90 than at 80 per cent relative humidity.

Roots inoculated in the same manner as the foregoing and dried with an electric fan were stored at relative humidities of 95, 90, 80, and 70 per cent at a temperature of 6.5° C. Those stored at 90 and 95 per cent relative humidity became infected, whereas those stored at 70 and 80 per cent did not. The percentage infected at 95 per cent relative humidity was higher than that at 90 per cent.

The relative susceptibility of the different varieties to bacterial soft rot and *Botrytis* has already been discussed, but it remains to point out that there is considerable variation in the percentage of infection with both diseases in the two lots stored at 0° to 2° and that they are not parallel. It is believed that this variation is normal and not due to the difference in container or position.

There is some variation in the percentage of infection of the different varieties at a particular temperature by *Fusarium* and *Penicillium*, but this variation is obviously affected by infection by the other pathogenes, nor is the variation always in the same direction at the other temperatures. Taking into consideration the total infection and the results in the last column, it is quite evident that no one variety is distinctly more resistant to disease than another.

Kristofferson (7), working with the Parisian carrot, Guérande, two strains of Nantes, and two of Saint Valery, not only found a difference in susceptibility to "winter rot," but discovered also that this susceptibility was correlated with the invert sugar content. For instance Guérande, with an invert sugar content of 4.53 per cent, showed 84.6 per cent rot, as compared with Saint Valery, strain B, with an invert sugar content of 2.43 per cent, which showed only 5 per cent rot.

These results are difficult of interpretation, (1) because the storage temperatures and other conditions of storage are not given and (2) because "winter rot" is rather a general term. At any rate, no such marked difference in susceptibility was found in these experiments, in these or other varieties tested. If the invert sugar content governs the susceptibility of a particular lot of carrots in any way, this susceptibility can not be expected to remain constant from year to year, for Hasselbring (5) found a greater variation in the invert sugar content of the same variety for different seasons than among the different varieties for the same season.

#### EFFECT OF HUMIDITY ON SHRIVELING

Carrot roots of all varieties are very susceptible to drying and shriveling, and the range of humidities at any particular temperature at which it is possible to maintain a desired turgor is relatively narrow.

At relative humidities of 90 and 95 per cent and a temperature of 6.5° C (Table 35)<sup>12</sup> all the roots remained firm during 104 days of storage, whereas there was considerable shriveling at both 70 and 80 per cent relative humidity (much more at 70 than at 80 per cent) after both 47 and 104 days of storage. At 70 per cent relative humidity there was a marked increase in shriveling as the storage period was prolonged. The data show a smaller percentage of shriveling at 80 per cent relative humidity after 104 days of storage than after 47 days. The numbers used in the two counts may have made some difference; otherwise there is no evident explanation of this discrepancy.

Although no data are available on the relation of humidity to shriveling at temperatures near 0° C, which is the most desirable temperature for the storage of carrots, experience shows that the relative humidity should range from 90 to 95 per cent.

<sup>12</sup> The data recorded in Table 35 were obtained from the same experiment as those given in Tables 6 and 27.

these varieties at temperatures of 0° to 2° C the only infection by *R. nigricans* observed, aside from that reported for the Danvers Half Long variety, occurred during the season 1926-27 at a temperature of 10° in the Rouge demi-longue de Danvers variety. The slight and rare infection in Danvers Half Long and Rouge demi-longue de Danvers and the absence of infection in all the other varieties indicate that all varieties are normally resistant to decay by *R. nigricans* at temperatures from 0°-2° to 15.5°

Sixteen varieties of carrot, dipped in a spore suspension of *Rhizopus tritici* and stored at 30° C or inoculated by the well method and stored at 23°, were very susceptible to infection and decay. No marked differences in susceptibility to this fungus were found.

That *Bacillus carotovorus* is constantly associated with carrots is indicated by the fact that a certain percentage of roots will invariably become infected if stored at temperatures ranging from 20° to 25° C, if the relative humidity is high (90 to 95 per cent).

Wounding was found to increase slightly the percentage of infection at temperatures ranging from 17.5° to 24° C, however, it was not essential to infection, a large percentage of infection occurring in its absence.

The extreme temperature limits at which infection of inoculated carrots by *Bacillus carotovorus* has been observed are 0° to 2° and 36.5° C. In only 1 out of 9 seasons of normal storage of the Danvers Half Long variety and in 1 out of 4 seasons of storage of several other varieties has bacterial soft rot been observed at a storage temperature of 0° to 2°. Infection has occurred in only 1 out of 3 seasons of normal storage of several varieties at 4.5°. Storage trials with the Danvers Half Long variety at temperatures near 4.5° have failed to yield infection by *Bacillus carotovorus*. In roots that were sound when stored, very little infection has ever been observed at temperatures below 10°.

There was little, if any, apparent difference in the time required for infection to occur in carrots stored at temperatures ranging from 21.5° to 36.5° C. As the temperature was lowered from 21.5° to 12.5° the time required for infection to develop increased, at 12.5° no infection occurring in 27 days and 15 per cent in 51 days.

The inclusion in the storage stock of carrots decaying with *Bacillus carotovorus* insures a more uniform distribution of the decay over a wider range of temperatures than occurs in the storage stock of roots free from such contamination.

In carrots inoculated by the well method the maximum temperature at which infection occurred within three days was 29° C., the optimum temperature for decay was 24.5°; and the minimum temperature for infection in seven days was 5°.

Taking into consideration the three types of experiments employed, the optimum temperature for decay is about 25° C.

The results regarding the influence of humidity on infection of carrots by *Bacillus carotovorus* are too incomplete and indecisive to make it possible to draw any conclusions from them.

Seventeen varieties of carrots have been found readily susceptible to infection and decay by *Bacillus carotovorus*. Although some variation in the percentage of infection and in the quantity of decay has been found in the different varieties in a given experiment, this variation

By storing dry roots, contaminated but not infected with *Sclerotinia sclerotium*, at a temperature of 0° and relative humidities ranging from 85 to 90 per cent it is believed possible greatly to inhibit infection.

Fourteen varieties of carrots have been found readily susceptible to infection by *Sclerotinia sclerotium*. No marked difference in susceptibility was observed among them.

*Rhizopus tritici* and *R. nigricans* are the only species of *Rhizopus* obtained from a large number of isolations from carrots subjected to a variety of temperatures and other storage conditions. Although it is possible that other species may decay carrots under special conditions, they do not do so normally under Washington conditions.

The extreme temperature limits at which infection of carrots by *Rhizopus* has been obtained in uninoculated roots are 0° to 2° and 44° C. Infection is always relatively heavy and occurs within a period of four days at temperatures above 30°. At temperatures below 30° infection is always relatively light, and the time required for it to occur is much longer. Below 20° infection is erratic in its occurrence, often not occurring at all during the marketable life of the roots and never in large amounts. In only two tests has infection been observed at temperatures below 12°, namely, at 0° to 2° and 10° in the season of 1926-27 and at 7° and 10° in the season of 1925-26 (Table 32 and unpublished data), and then it was very limited.

The time required for *Rhizopus* infection to develop in uninoculated roots increased with the lowering of the temperature below 36.5° C. Only 4 per cent occurred at 12.5° in 51 days.

The extreme temperature limits at which infection of uninoculated carrots by *Rhizopus tritici* has been obtained are 19° and 44° C, in roots inoculated by the well method infection has been obtained at 5°.

The extreme temperature limits at which infection of uninoculated carrots by *Rhizopus nigricans* has been obtained are 0° to 2° and 19° C. Infection by this pathogene in roots inoculated by the well method has occurred at 28.5°, and possibly higher. The upper temperature limit for infection by this pathogene has not been accurately determined, because of complications produced by infection by *Rhizopus tritici*.

Infection by *Rhizopus nigricans* is normally rare and small in amount, indicating that carrots are highly resistant to attack by this pathogene.

The optimum temperature for decay of carrots inoculated by the well method with *Rhizopus tritici* was about 33.5° C, and for those inoculated with *Rhizopus nigricans* the optimum temperature was about 28°. Roots inoculated by being dipped in a spore suspension of either pathogene, or both, may show a small increase in the percentage of infection within certain temperature limits and a lower limit of infection. However, such is not always the case, nor is the effect ever marked.

Wounding generally tends to increase the percentage of infection by *Rhizopus* and to drop the lower limit of infection, but the effect is not marked nor is wounding indispensable to infection.

Seventeen varieties of carrots were found to be susceptible to infection and decay by *Rhizopus nigricans* when the roots were inoculated by the well method. During three years of normal storage of

life of the carrots at the various temperatures, the following diseases may be expected to develop *Rhizopus* soft rot, bacterial soft rot, *Fusarium* rot, *Penicillium* rot, and *Botrytis* rot. The percentage of roots infected and the temperature limits at which infection by the various pathogenes takes place will vary somewhat with the stock stored. The amount of decay produced by one pathogene often influences the amount produced by another.

In a certain experiment in which carrots were stored at temperatures ranging from 1° to 36.5° C (Table 30), the temperature limits within which decay occurred were as follows: *Rhizopus* soft rot, 12.5° and 36.5°, bacterial soft rot, 12.5° and 36.5°, *Fusarium* rot, 6.5° and 36.5°, *Botrytis* rot, 1° and 8°, and *Penicillium* rot, 6.5° and 36.5°. *Penicillium* rot occurred at only one temperature (19°) between 12.5° and 36.5°, and mostly at 6.5° to 12.5°, inclusive.

The market life of carrots, with reference to the development of diseases, increases as the temperatures are lowered below 36.5° C.

A factor other than infection and decay that greatly limits the life of carrots at temperatures from 6.5° to 31° C. is sprouting. No sprouting occurred on roots stored at 36.5° for 12 days. Sprouting occurred at 31°, 24.5°, 21.5°, and 19° in 5 days, at 15.5° and 12.5° in 12 days, at 8° in 19 days, and at 6.5° in 27 days. No sprouting occurred at 1° and 2° in 113 days.

The diseases that developed in the Danvers Half Long variety at one temperature or another during four different years of storage at temperatures ranging from 0°-2° to 15.5° C. were: Bacterial soft rot, black rot, *Fusarium* rot, *Penicillium* rot, *Rhizopus* soft rot, and *Botrytis* rot. This range of temperatures coincides approximately with the limits one might expect to find in common storage.

The losses during five different years of storage of the Danvers Half Long variety at temperatures fluctuating between 0° and 2° ranged from 2 to 20 per cent. The relative humidities varied during the several seasons from 85 to 91 per cent and the storage periods from 50 to 201 days.

A survey of the diseases that developed in 17 varieties of carrots stored at temperatures ranging from 0°-2° to 15.5° C. during the season 1926-27 and in most of the varieties during four other seasons showed that all the varieties were susceptible to *Fusarium* rot and to *Penicillium* rot in addition to the diseases discussed hitherto. No marked difference in susceptibility was found.

No shriveling occurred in carrots stored at a temperature of 6.5° C. and at relative humidities of 90 and 95 per cent, whereas considerable shriveling occurred at relative humidities of 70 and 80 per cent.

The environmental conditions regarded as most favorable for storage of carrots are a temperature of 0° C. and a relative humidity of 90 to 95 per cent.

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was not always paralleled in a second experiment, and differences in susceptibility have not been sufficiently consistent to give any variety an outstanding position in regard to susceptibility or resistance. The *Blanche lisse* demi-longue variety may be slightly more susceptible than the others.

The uniformity with which *Botrytis* rot develops when carrots are stored for long periods at temperatures ranging from 0° to 5° C indicates that *Botrytis cinerea* is always present in carrot storage houses.

Infection of carrots by *Botrytis cinerea* is aided by the presence of nutrient media as used in the well method of inoculation. Normal infection seems to be associated with the collapse of certain tissues such as tops, secondary roots, wounded areas, and the fine tip ends of taproots. Most of the infection occurs in the last-mentioned way. Aside from the infection that occurs through secondary roots, the fine tip ends of taproots, and the tops, infection takes place almost exclusively through wounds, more readily through fresh wounds than old ones, and rarely if ever through the uninjured skin.

The cardinal temperatures for the growth of *Botrytis cinerea* on culture media are approximately as follows: Maximum, 32° to 35° C, optimum, 24°, and minimum, about 0°.

*Botrytis* rot in carrots taken directly from the field and stored at various temperatures is usually confined to temperatures below 15° C. If carrots are placed at higher temperatures after having been stored at a temperature near 0° for a time, the range may be extended to higher temperatures.

Infection of carrots by *Botrytis* takes place slowly in roots taken directly from the field and stored at temperatures ranging from 0° to 2° C; that is, the percentage of infection is relatively small during the first 50 days and decay is, as a rule, in its initial stage. Infection and decay increase slowly as the storage period extends beyond 50 days.

The widest range of temperatures at which *Botrytis cinerea* infection has been obtained on carrots extends from 0°-2° to 24.5° C, as compared with a temperature range extending from 0° to 32° for growth of *B. cinerea* on carrot agar. The maximum amount of decay on carrots was obtained at 22.5°, which is very close to the optimum temperature (23.5°) obtained for growth of the pathogen on carrot agar. The difference may be accounted for by the difference in temperatures employed in the two experiments.

In carrots taken directly from the field and stored without inoculation at a temperature of 6.5° C for 104 days there was very little difference in the percentage of infection at relative humidities of 80, 90, and 95 per cent. There was a drop in the percentage of infection as the relative humidity fell from 90 to 70 per cent. There was no infection at a relative humidity of 70 per cent in uninoculated and dried inoculated roots and only 2 per cent in roots stored when wet, indicating that 70 per cent relative humidity inhibits infection. This humidity, however, is too low for the proper storage of carrots, since it causes them to shrivel.

Eighteen varieties of carrots have been found to be susceptible to infection and decay by *Botrytis cinerea*. No consistent differences in their susceptibility to this fungus were found.

If carrots free from contamination by *Sclerotinia sclerotiorum* are stored at temperatures ranging from 0° to 40° C. and at relative humidities of 90 per cent and above for a period covering the market

# THE SPOILAGE OF DRESSED DUCKS BY SLIMINESS<sup>1</sup>

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## INTRODUCTION

A select and extensive market has been created for the sale of what the trade designates as green ducks, squab ducklings, or Long Island ducks. These ducks are from 5 to 10 weeks of age and weigh from 3 to 5 pounds. The immaturity of their tissues renders them very soft and tender, with the result that they spoil rather rapidly, much more so than mature carcasses.

When squab ducklings are shipped in crushed ice or ice water, very little spoilage results, but when they are shipped moist in containers which permit them to come in contact with the air, they become slippery, particularly beneath the wings. In extreme cases the entire carcass is covered with slime so thick that it is difficult to pick up the duck with the hands. Accompanying the slime formation is a markedly offensive odor. In the early stages decomposition is purely superficial and washing in a solution of sodium carbonate will remove all odor and slime. The skin, however, has a roughened appearance, which renders the duck unfit for market. The internal organs and other tissues do not show any decomposition or odor even in pronounced cases of sliminess.

Slipperiness will develop in from one to two days even when carcasses are kept constantly at ice-box temperature (50° F.). Carcasses stored in meat-market ice-cooled refrigerators spoil with marked regularity. Mechanical refrigeration at a temperature of 40° F. prevents sliminess but causes a drying out of the skin, which is accompanied by discoloration and cracking. The resulting product is as unmarketable as the slimy ducks, owing to the objectionable appearance of the carcasses.

This type of spoilage first came to the attention of the writer when a Michigan packing plant began shipping ducks in individual cartons direct to the consumer. This plant had previously shipped its entire output in crushed ice without any evidence of slime formation. At the time that sliminess was observed in ducks shipped in paper cartons, those shipped in crushed ice were free from this trouble. All the ducks were dressed in the same plant at the same time under identical conditions, the only difference in the preparation was in the mode of shipping. Further investigation revealed the fact that sliminess is of frequent occurrence in squab-duckling packing plants and, furthermore, that this condition never occurs when the ducks are shipped in crushed ice.

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disinfectant that would act in the presence of excessive quantities of organic matter was necessary, also it should have a low toxicity, as it would be undesirable to add any substance that would injure the food value of the duck.

Sodium hypochlorite was finally selected because it meets these requirements and because of its adaptability and low cost. Since much dirt and contamination enter the scalding tanks, it was believed that any attempt to use continuous disinfection in these tanks would not be feasible. Instead, a system of disinfection in the final ice-water storage tanks was attempted. In these tanks sodium hypochlorite in doses of 100 parts per million of available chlorine was added. Two tanks were used, one was treated with neutral sodium hypochlorite and the other with an alkaline sodium hypochlorite. The dressed ducks were stored in these tanks for approximately 24 hours, after which they were removed, packed in cartons, and stored in an ice-cooled refrigerator. Untreated ducks were similarly stored as controls. The results of this experiment are presented in Table 1.

TABLE 1 —Results of disinfecting dressed ducks in chlorinated ice water

Duck No	Treatment	Condition of carcass after—	
		6 days	10 days
1.....	None.....	Decidedly slimy and discolored.....	Poor
2.....	do.....	do.....	Do
3.....	Neutral sodium hypochlorite.....	Slimy and discolored.....	Fair
4.....	Alkaline hypochlorite.....	Excellent.....	Best
5.....	do.....	Slightly slimy on legs only.....	Good
6.....	do.....	Slimy and discolored.....	Fair

At the end of six days, only one duck (No. 4) that was treated with neutral sodium hypochlorite was really marketable as a product of the highest quality. The control ducks (untreated) were unmarketable at the end of six days. At the end of 10 days all the ducks were unmarketable; however, the treated ducks were in better condition than the untreated ones. The experiment demonstrated that treatment with sodium hypochlorite in ice water will extend the keeping period but that it does not eliminate ultimate spoilage by slime formation.

To determine the efficiency of hypochlorite for killing the organisms causing sliminess, a series of laboratory experiments was made in which a technic was used similar to that employed in determining the phenol coefficient of a disinfectant. Temperatures of 10°, 20°, and 37° C. were used with exposures of 1, 5, 10, 30, 60, and 120 minutes. The culture was a 48-hour agar slant containing spores. Two series of tests were made, one with 0.1 cc of culture suspension and the other with 0.01 cc of suspension. Three strengths of hypochlorite were used, namely, 25, 50, and 100 parts per minute of available chlorine. The results are presented in Table 2. Only the data for 0.1 cc of culture suspension are given, since the results with 0.01 cc were the same.

## EXPERIMENTAL STUDIES

Bacteriological examinations made from slimy ducks revealed several spore-forming bacilli. The predominating organism was a large, nonmotile, Gram-positive, spore-forming bacterium with a large capsule. The organism has all the characteristics of *Bacillus mesentericus* except motility. It appears to be either an undescribed form or a nonmotile variant of *B. mesentericus*. It was obtained in practically pure culture from the ducklings examined.

In the killing plant cultures were made from all the tanks and tables in or upon which the ducks were handled. The same organism was isolated from each of the following places: The scalding tank (140° F.), plumping tank (212°), chilling tank (50°), ice-water tank, dressing tables, floor, feet of live ducks entering the plant, and shelves in ice chest. The organism was not found in the water supply used at the killing plant.

To check further the significance of the organism as a causative factor in producing sliminess, five live ducklings from this plant were dressed at the college poultry plant, sterilized containers being used for scalding and cooling. The college dressing plant was completed shortly before this experiment was conducted, so all the equipment was new. In order to be sure that all contamination was excluded, the tanks were carefully cleaned with a strong solution of sodium hypochlorite, and 100 parts per million of chlorine in the form of sodium hypochlorite was added to the scalding water to destroy any organisms that might be present on the ducks. The method of dressing was exactly the same as that used at the duck-packing plant. After the ducks were dressed and cooled, two were immersed for five minutes in a suspension of the encapsulated organism. The remaining three were kept as controls. All the ducks were then placed in paper cartons and immediately stored in an ice-cooled refrigerator at a temperature of 50° F. A duck dressed in the packing plant was stored in the same refrigerator. Each day for a period of eight days the ducks were removed from the refrigerator and examined for slime formation. Within two days the duck dressed at the plant was too slimy to be marketable. On the sixth day the inoculated ducks were slippery under the wings, but the control ducks were free from spoilage. On the eighth day the inoculated ducks were decidedly slimy, the slime then covering the entire carcass. An offensive odor was present. One of the controls was slightly slippery under the wings, but the other two were still quite normal. The same organism was recovered from all the ducks that showed evidence of slime formation. The results obtained clearly indicate that the organism isolated is a causative factor in the production of sliminess and that the source of contamination is the utensils used in the dressing and cooling processes. Thus it appeared that a clean plant might solve the problem. Accordingly, means by which this might be accomplished were considered.

The use of a chemical disinfectant appeared to be the best procedure, since the organisms apparently survive boiling, as indicated by their isolation from the plumping tank at a temperature of 212° F. A

after 24 hours' immersion in 100 p p m of available chlorine to determine whether the chlorine had impaired their value as food. The treated ducks showed no injurious effects from the treatment. Nevertheless, it was thought that a minimum amount of chlorine should be used. To determine what the minimum should be, experiments were conducted with small amounts of residual chlorine. When 3.5 p p m of available chlorine was used, all the culture tubes showed growth up to 30 minutes, the longest period of exposure employed. However, there was a decided reduction in the number of bacteria as evidenced by the bacterial counts. Apparently the most effective results were obtained with a minimum of 25 p p m of available chlorine, which was sufficient for complete disinfection.

A second method of control was attempted in which the ducks were immersed in a disinfecting or antiseptic solution of sufficient strength to assure a retention of the antiseptic substance on the duck until it reached the consumer. The disinfectant used had to be one free from objectionable odor and taste. Sodium hypochlorite, sodium borate, sodium salicylate, and sodium chloride were tried. The sodium hypochlorite when used in strong solutions produced a medicinal odor in the paper carton that would be objectionable to the consumer. The sodium borate and sodium salicylate were not sufficiently antiseptic to retard putrefaction materially, hence these compounds were discarded. Dipping the ducks in a saturated solution of sodium chloride, however, eliminated the formation of slime. In this procedure the chemical is not used up by combining with the organic matter, as is the case with sodium hypochlorite. Instead, as the moisture evaporates from the surface of the duck, the salt becomes more concentrated and hence more active as an inhibiting agent. Sodium chloride, moreover, has no objectionable features as a food preservative.

To determine the concentration of salt necessary to inhibit the growth of the bacteria, different quantities of sodium chloride were added to plain nutrient broth into which the various cultures were planted by adding a loopful of a 24-hour broth suspension. The results are presented in Table 4.

TABLE 4.—*Inhibitive action of sodium chloride upon the growth of microorganisms causing slime formation in dressed ducks*

Microorganism No.	Control	Negative or positive results when using a concentration of salt of—		
		2 per cent	5 per cent	10 per cent
1.....	+	+	+	—
6.....	+	+	+	—
10.....	+	+	+	—

The above table shows that a concentration of 10 per cent of sodium chloride is necessary to suppress the development of the bacteria. That the action is purely inhibitive and not germicidal was demonstrated by making plate counts from these tubes at various intervals up to 20 minutes. Although counts were made from salt concentrations of 2, 5, and 10 per cent, only the last are presented, as similar results were obtained with the 2 and 5 per cent concentrations. The data are shown in Table 5.

TABLE 2—Killing power of sodium hypochlorite as determined on a 0.1 c c culture suspension of the organism causing sliminess in dressed ducks

Available chlorine	Temperature of exposure	Negative or positive results after exposure of—					
		1 minute	5 minutes	10 minutes	30 minutes	60 minutes	120 minutes
<i>P p m</i>	<i>° C</i>						
25	10	+	+	+	+	+	+
50		+	+	+	+	+	+
100		+	+	+	+	+	+
25	20	+	—	—	—	—	—
50		+	—	—	—	—	—
100		+	—	—	—	—	—
25	37	+	—	—	—	—	—
50		+	—	—	—	—	—
100		+	—	—	—	—	—

The data indicate that sodium hypochlorite in ice water at all strengths tested was ineffective during the periods of exposure used. At 20° and at 37° C the chlorine was comparatively active, causing complete destruction of all vegetative cells and spores in five minutes or less.

In a later examination of slimy ducks, three other organisms were isolated—a noncapsule-forming spore producer, a coccus, and a nonspore-forming rod. These organisms were selected because the odor of the cultures was identical with that found on the slimy ducks. Their resistance to chlorine was studied. The results are presented in Table 3.

TABLE 3—Killing power of sodium hypochlorite as determined on three microorganisms found on slimy ducks

Culture No. <sup>a</sup>	Available chlorine	Temperature of exposure	Control	Negative or positive results after exposure of—					
				1 minute	5 minutes	10 minutes	15 minutes	20 minutes	30 minutes
	<i>P p m</i>	<i>° C</i>							
1.....	50	22	+	—	—	—	—	—	—
6.....			+	—	—	—	—	—	—
10.....			+	—	—	—	—	—	—
1.....	25	22	+	+	+	—	—	—	—
6.....			+	—	—	—	—	—	—
10.....			+	—	—	—	—	—	—
1.....	50	7	+	—	—	—	—	—	—
6.....			+	—	—	—	—	—	—
10.....			+	—	—	—	—	—	—
1.....	25	7	+	—	—	—	—	—	—
6.....			+	—	—	—	—	—	—
10.....			+	—	—	—	—	—	—

<sup>a</sup> Culture No. 1, a noncapsule-forming, spore-forming bacillus, No. 6, a Gram+micrococcus, No. 10, a Gram+nonspore-forming rods.

The three organisms were readily killed by chlorine in ice water in concentrations of 25 p p m of available chlorine. The fact that one of the organisms was a spore former indicates that chlorine does have a decided germicidal value at low temperatures, and that in spite of its inability to destroy all the capsulated spore formers present, it can reduce to a marked degree the amount of contamination carried on the surface of the duck.

Although chlorine in the quantities used had no effect on the appearance of the ducks, a careful examination was made of ducks

# EFFECT OF FERTILIZERS ON THE CHLORINE CONTENT OF THE SAP OF CORN PLANTS<sup>1</sup>

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## INTRODUCTION

In a recent paper (14)<sup>3</sup> the writer reported some preliminary results of an investigation which showed that the composition of the sap of corn (*Zea Mays* L.) plants was markedly affected by the addition of fertilizers to the soils on which the plants were grown. At the time these results were reported, the sap samples had been analyzed for nitrate nitrogen, total phosphorus, and total potassium only. In an effort to explain certain data obtained in the corn-sap studies now under way at the Virginia station, it seemed desirable to determine whether the various sap samples contained large, medium, or small amounts of some of the fertilizer constituents that are usually given minor consideration in the use of fertilizers. Accordingly, the sap samples were analyzed for the materials of interest. The purpose of the present paper is to present the data from these analyses that concern mainly the effect of chlorine-carrying fertilizers on the chlorine content of the sap of corn plants as found in a 4-year study under field conditions.

## MATERIALS AND METHODS

The plants from which sap was extracted for this investigation were grown on the "rotation with fertilizer" plots at the Virginia Agricultural Experiment Station, Blacksburg, Va. Since a complete description of these plots and their management has been given in a previous report (14), only the more salient features will be repeated here. The experiment was started in 1909 on a Hagerstown silt loam soil of moderate fertility, and embraced four series of 13 plots each. These four series of plots, designated as A, B, C, and D, carry in rotation the following crops: First year, corn, second year, wheat, third and fourth years, hay (mammoth clover, redtop, and timothy). The kinds and the combinations of fertilizers applied to the various plots are indicated in Table 1. From 1909 to 1914, each fertilizer treatment was applied to the entire plot. In the latter year, however, all plots were divided in half. Since 1914, with the exception of farm manure, the quantity of fertilizer that was formerly applied to the entire plot has been applied to the south half of each plot, thereby doubling the former rate of application on this part of each plot, except the manure plots. From 1917 to the present time, the rates of application per acre have been as follows: Dried blood, 308 pounds, ammonium sulphate, 200 pounds, rock phosphate, 219 pounds, superphosphate, 438 pounds; muriate of potash, 200 pounds; and manure,

<sup>1</sup> Received for publication Dec. 28, 1931, issued July, 1932.

<sup>2</sup> The writer is indebted to the Departments of Agricultural Chemistry and of Botany and Plant Pathology for laboratory facilities in making the chemical analyses reported in this paper.

<sup>3</sup> Reference is made by number (italic) to Literature Cited, p. 930.

TABLE 5—Effect of a 10 per cent solution of sodium chloride on the count of certain microorganisms causing slime formation in dressed ducks

Microorganism No	Control count	Count after exposure of—				
		1 minute	5 minutes	10 minutes	15 minutes	20 minutes
1.....	133	261	201	200	192	149
6.....	186	253	256	219	246	251
10.....	235	323	245	266	261	254

It will be observed that no reduction occurred in the number of bacteria. In fact, there was an apparent increase, probably owing to the repeated shaking of the tubes at the time of sampling which caused a dispersion of clumps of bacteria.

### DISCUSSION

As the data indicate, two methods of controlling slipperiness in squab ducklings may be used, namely, sanitation and preservation. The experiments demonstrate that the use of clean, sterile utensils with clean, sterile, scalding, plumping, and chilling waters will result in a product that develops sliminess very slowly, the period of delay being sufficient for the usual marketing. However, the maintenance of such sanitary conditions is not practicable in the usual killing plant and is prohibitive in cost, so it becomes necessary to use chemical preservatives along with economical and practical sanitary measures. It has been demonstrated that a disinfection of the carcass with sodium hypochlorite in the final ice-water storage tank materially lessens the amount of contamination and thus delays spoilage. A final dipping of the finished product in a concentrated salt solution results in covering the carcass with a thin film of salt brine that inhibits the growth of the slime-producing bacteria and thus prevents spoilage. In practice the treatment of the ducks by immersing in ice water containing sodium hypochlorite, followed by dipping in saturated salt brine just previous to packing for shipment, has given very satisfactory results. It may be that the antiseptic action of the salt brine is sufficient and that the addition of sodium hypochlorite to the storage tank is unnecessary.

### SUMMARY

Slipperiness in squab ducklings is caused by bacteria.

Four microorganisms were isolated from slimy ducklings. The chief microorganism involved in slipperiness is a spore-forming encapsulated bacillus closely resembling *Bacillus mesentericus*. This organism was found on all utensils and exposed surfaces in the killing and dressing rooms of the duck-packing plant investigated.

Slime formation was induced by inoculating ducklings with pure culture of this organism.

The addition to the storage tanks of 100 p. p. m. of available chlorine in the form of sodium hypochlorite delayed the formation of slime.

Dipping the dressed ducklings in saturated salt brine stopped the formation of slime.

In most cases, the data from individual years do not deviate greatly from the averages just considered. It is evident from these data, therefore, that the application of chlorine-carrying fertilizers increased greatly the chlorine content of the sap of corn plants.

TABLE 1. Chlorine content of sap extracted from corn plants grown on plots receiving different fertilizer treatments

[Values are in milligrams of chlorine per cubic centimeter of sap]

Plot No	Treatment <sup>a</sup>	Chlorine in sap extracted on—					Average August samples	Average all samples
		Aug. 29, 1928	Sept. 25, 1928	Aug. 30, 1929	Aug. 30, 1930	Aug. 27, 1931		
		Mg	Mg	Mg	Mg	Mg	Mg	Mg
2D <sub>1</sub> <sup>b</sup>	O		0.14	0.14	0.18	0.07	0.13	0.13
2D <sub>2</sub>	Ps	0.12	0.09	0.15	0.16	0.17	0.15	0.14
3D <sub>1</sub>	O	.31	.26	.15	.19	.18	.22	.22
3D <sub>2</sub>	N, Ps, K	3.21	2.86	1.32	1.20	1.44	1.82	2.02
4D <sub>1</sub>	Check	.19	.40	.18	.18	.12	.17	.21
4D <sub>2</sub>	N(ams), Pr.	.20	.13	.16	.11	.06	.13	.13
5D <sub>1</sub>	O	.17	.36	.19	.26	.46	.27	.29
5D <sub>2</sub>	Ps, K	2.62	2.54	2.39	.96	2.26	2.11	2.19
6D <sub>1</sub>	O	.47	.85	.35	.26	.45	.38	.48
6D <sub>2</sub>	K	2.59	2.13	1.31	1.06	1.74	1.68	1.77
7D <sub>1</sub>	O	.21	.78	.35	.17	.25	.25	.35
7D <sub>2</sub>	N, K	2.48	2.89	1.26	1.22	1.11	1.52	1.79
8D <sub>1</sub>	O	.01	.37	.13	.12	.08	.09	.15
8D <sub>2</sub>	N, Ps	.16	.31	.18	.15	.11	.15	.18
9D <sub>1</sub>	O	.19	.29	.17	.15	.08	.15	.18
9D <sub>2</sub>	N	.13	.15	.17	.14	.12	.14	.14
10D <sub>1</sub>	O	.11	.15	.12	.11	.10	.11	.12
10D <sub>2</sub>	M	2.63	2.26	.66	.50	.70	.97	1.23
11D <sub>1</sub>	Check	.13	.29	.12	.10	.17	.13	.16
11D <sub>2</sub>	M, Pr	2.57	2.98	.78	.60	.58	1.13	1.50
12D <sub>1</sub>	O	.17	.35	.09	.09	.10	.11	.16
12D <sub>2</sub>	M	.76	1.13	.36	.58	.51	.55	.67
13D <sub>1</sub>	O	.20	.11	.09	.12	.07	.12	.12
13D <sub>2</sub>	M, Ps		2.42	.59	.95	.61	.72	1.14
14D <sub>1</sub>	O		.36	.18	.18	.16	.17	.22
14D <sub>2</sub>	Pr	.14	.18	.05	.16	.13	.12	.13

<sup>a</sup> Check, no treatment since 1909, O, no treatment since 1914, N, nitrogen from dried blood, N(ams), nitrogen from ammonium sulphate, M, manure, P, rock phosphate, Ps, superphosphate, K, muriate of potash.

<sup>b</sup> All plots on range D in 1928, range C in 1929, range B in 1930, and range A in 1931.

The failure of the saps from the manure plots to contain as much chlorine as those from plots which received muriate of potash raises the question whether these differences in chlorine content might possibly be related to differences in the amounts of chlorine supplied by the two fertilizers. The manure which has been used in the past on these plots has been produced by work horses kept at the experiment station. These animals are usually bedded with cereal straws and fed on various grass and legume hays. Van Slyke (19, p. 83) reports the chlorine content of oat straw as being 0.31 per cent. This figure is presumably for air-dry straw, and it would be logical to expect fresh manure to show a much lower chlorine content when analyzed. This was confirmed by an analysis of four samples collected individually and at random from the manure now in storage at the experiment station barns, which showed chlorine contents of 0.13, 0.15, 0.18, and 0.18 per cent, respectively, on the wet basis. The moisture content of the four samples was 72.4, 67.6, 67.5, and 68.3 per cent, respectively. On the basis of these analyses a ton of manure contains approximately 3.2 pounds of chlorine. Since the manure was applied at the rate of 16 tons per acre in four years, the plots received approximately 51

16 tons once in four years, except plot 12, which received 4 tons annually. All applications were made annually except as noted otherwise.

The sap samples were obtained from 15-inch sections of the stalks immediately above the surface of the soil. These sections were cut into small pieces approximately an inch in length, and subjected to a pressure of 6,500 pounds per square inch in a small Carver laboratory hydraulic press. The sap samples thus obtained in 1928 and 1929 were clarified immediately with carbon black and then stored in a refrigerator at  $-7^{\circ}\text{C}$  until removed for analysis. The samples obtained in 1930 and 1931 were not clarified. A small amount of toluene was added to each sample to prevent fermentation.

The samples were collected during the last week in August each year. In 1928, a second set of samples was taken during the last week in September.

Chlorine content was determined as follows. 10 c c of sap were placed in an evaporating dish with 20 c c of a 5 per cent sodium-carbonate solution, evaporated to dryness, ashed at a very low temperature, taken up with water, and filtered. The filtrate was then titrated with a standard silver nitrate solution in the presence of a yellow light, potassium chromate being used as indicator. The titrations were made under a yellow light because the potassium-chromate indicator imparts a yellow color to the solution to be titrated and, hence, in order to sensitize the end point properly, it was desirable to have the solution and the light of the same or similar color and shade of color. A yellow 25-watt incandescent lamp was used as the source of light. When the chlorine analyses were first undertaken, considerable difficulty was experienced in getting the samples to ash completely at temperatures sufficiently low to prevent volatilization of the chlorides. This difficulty was overcome by moistening the incompletely ashed residue with water, redrying in an electric oven, and then returning the sample to the furnace. In some cases a second moistening was necessary for complete combustion.

## PRESENTATION, INTERPRETATION, AND DISCUSSION OF EXPERIMENTAL RESULTS

### EFFECT OF FERTILIZERS CONTAINING CHLORINE ON THE CHLORINE CONTENT OF THE PLANT SAP

The results obtained in this investigation show a number of interesting relations (Table 1 and fig 1). The most evident of these is the marked effect that chlorine-carrying fertilizers had on the chlorine content of the plant sap. Considering for the moment only the 4-year averages for the samples collected in August, it will be found that the plots which have received no chlorine at any time during the experiment (except in crop residues) yielded saps containing less than 0.20 mg of chlorine per cubic centimeter (200 parts per million). On the other hand, saps coming from plots which received muriate of potash annually, contained more than 1.60 mg of chlorine per cubic centimeter (1,600 p. p. m.), an increase of over 700 per cent. The manure plots yielded saps which contained between 0.50 and 1.10 mg of chlorine per cubic centimeter (500 to 1,100 p. p. m.). They were therefore distinctly intermediate between the saps from untreated plots and those from plots which received muriate of potash annually.

It is also clear that although muriate of potash increased the chlorine content of the plant sap more than manure did, the increase is not proportional to the amount of chlorine applied. The saps from the muriate of potash plots contained about twice as much chlorine as those from the manure plots, but this increase is far from being directly proportional to the 375 pounds of chlorine supplied by the muriate per rotation as compared to 51 pounds supplied by manure. It is not possible to account for this difference on the basis of differential rates of utilization of chlorine within the plants, because this element enters into practically no organic compounds occurring naturally in plants except the anthocyanin pigments. Hence, the amount of chlorine assimilated by corn plants must be very small, and differential utilization would bring about only small differences. It is possible, however, that the large amount of chlorine supplied by the muriate may have exceeded the capacity of the corn plant to absorb and retain this element. In this event, applications of chlorine larger than the amount necessary to extend the plant to its capacity in this respect, would effect no increase in the chlorine concentration of the sap.

EFFECT OF DATE OF SAMPLING ON THE CHLORINE CONTENT OF THE  
PLANT SAP

As previously pointed out, two sets of samples were collected in 1928, the first during the last week in August and the second during the last week in September. The data in Table 1 show that in 16 of the 23 comparisons the September samples contained more chlorine than the samples obtained from the same plots a month earlier. This indicates that in many of the plots chlorides continued to move into the plants until they were nearly mature. Similar results have been reported by Harris (3), who found that chlorides accumulated in the leaf-tissue fluids of cotton plants with the march of the season.

It is of interest also to compare the September movement of chlorides in the various plots with their respective fertilizer treatments. Where no chlorine has been applied since 1914, 75 per cent of the total number of saps show a higher chlorine content in September than in August. Where muriate of potash has been applied, however, 75 per cent of the total number of saps show a lower chlorine content in September. The saps from the manure plots show higher chlorine concentrations in the September samples.

The increases in concentration of chlorine in the saps from the untreated and manure plots and the decreases in the saps from the plots which received muriate of potash are difficult to reconcile. In the case of increases, it is natural to assume that the plants continued to absorb chloride ions during September in such quantities as to accumulate an observable increase. It is possible, however, that a part of the increases in chlorine concentration was caused by (1) a loss of water during maturation, or (2) by the movement of chlorine into the stalk from other parts of the plant. Desiccation incident to maturation is not believed to have had much influence, for there appeared to be as much or more sap in the stalks in September than in August. Furthermore, no appreciable differences were observed in the time of maturity of the corn on the various plots. The movement of chlorine into the stalks from other parts of the plant, par-

pounds of chlorine for each rotation Muriate of potash applied annually at the rate of 200 pounds per acre supplied approximately 375 pounds of chlorine in each 4-year period, nearly eight times as much as the manure furnished It is clear from these figures that

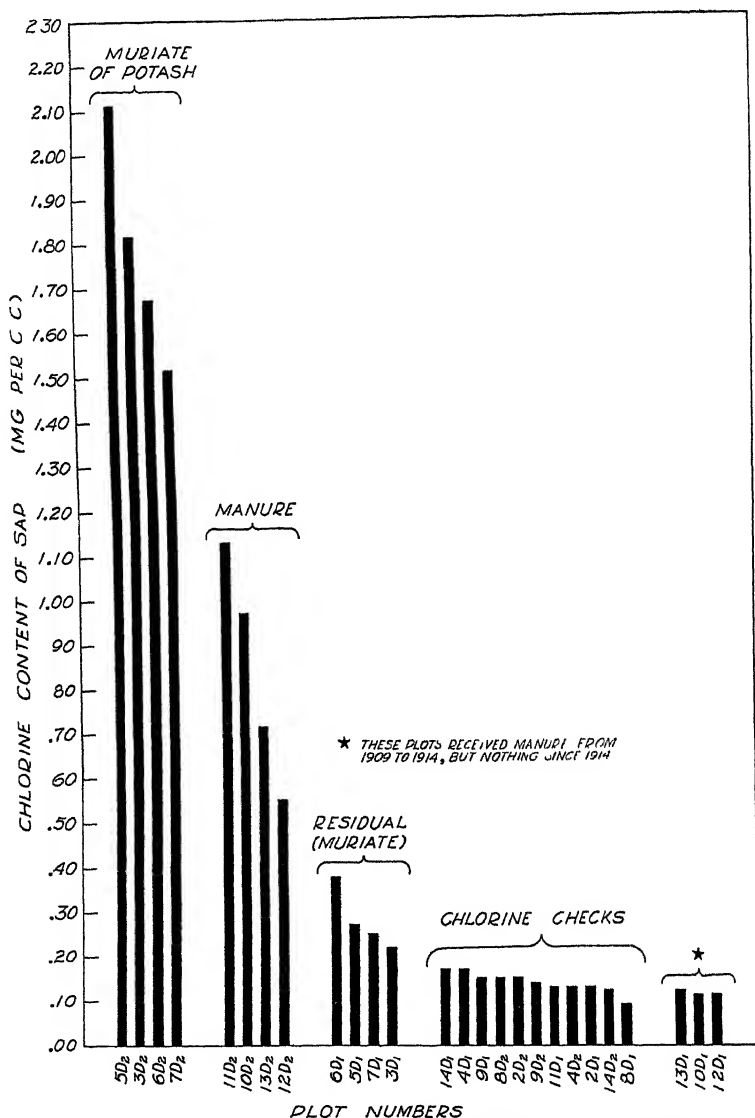


FIGURE 1—Chlorine content of sap of corn plants as affected by fertilizer treatment

the lower chlorine content of the saps from the manure plots was due to the smaller amount of chlorine supplied by the manure, and that the higher concentration in the sap from the muriate of potash plots was caused by the larger amount of chlorine supplied by the muriate.

more chlorine than the saps from check plots. As previously stated, these four plots received 100 pounds of muriate of potash per acre annually from 1909 to 1914. In 1914 they were divided into halves and the fertilized halves have since received the same amounts of fertilizer as previously applied to the entire plot, making the rate of application for muriate of potash 200 pounds per acre since 1914. The unfertilized halves have received no fertilizer since 1914. In view of the marked effect that muriate of potash exerted on the chlorine content of the plant sap when applied annually from 1909 to 1931 inclusive (fig. 1), it is evident that the saps from the untreated ends of plots 3, 5, 6, and 7 show the residual effects of the muriate of potash applied from 1909 to 1914. The saps from these plots also show the residual effects of the potassium applied in the muriate of potash (14, Table 2).

The saps from the untreated ends of plots 10, 12, and 13, which received farm manure from 1909 to 1914 but nothing since the latter date, show no residual effects of the chlorine supplied by the manure approximately 15 years ago. In fact, the saps from these plots contained slightly less chlorine than most of the check plots. These data, therefore, seem to be at variance with the generally accepted belief that organic fertilizers, especially farm manures, have residual effects of longer duration than do inorganic fertilizers (13, p. 614, 20, p. 221). It must be kept in mind, however, that the manure supplied less than one-third as much chlorine as the muriate of potash supplied. Furthermore, the manure plots have consistently returned the highest crop yields, and therefore have manifested a larger demand for chlorine than the other plots. Hence, the larger demand for chlorine occurred on the plots to which the smaller amounts were formerly applied. Over the intervening period of about 15 years this appears to have been sufficient to exhaust the chlorine which remained as residual material when the manure treatment was discontinued on these plots.

The annual yield data from the plots which formerly received muriate of potash show a strong tendency for the residual chlorine to be inversely proportional to crop growth (Fig. 2). The yielding power of these plots has thus far been determined largely by the degree of fertilization, i. e., whether a complete fertilizer was applied or one carrying but one or two of the three major fertilizer constituents, nitrogen, phosphoric acid, and potash. In general, the plots which have received but one constituent have given the lowest yields, those which have received two constituents have returned fair yields, while the plots which have received a complete fertilizer have given the highest yields. Of the plots showing residual chlorine, plot 3, which received a complete fertilizer from 1909 to 1914, shows the least residual effect. This plot has a crop yield index of 142 since 1914. Plot 6, on the other hand, which formerly received muriate of potash only, has a yield index of only 104 and shows the strongest residual effect. Plots 5 and 7, each of which formerly received fertilizers carrying two of the three major constituents, show intermediate residual effects. The yield index of 125 for plot 5 is also intermediate, but the index of 101 for plot 7 is low. On the whole, these data show that the more complete fertilization previous to 1914 has caused greater crop growth since fertilization was discontinued, which, in turn, resulted in

ticularly from the maturing leaves, remains as a possible partial explanation, for it is well known that when plants ripen the salts held in the sap have a tendency to migrate from the dying to the living tissues. This possibility is strengthened by the fact that approximately nine-tenths of the chlorine in cereal plants is contained in the stem tissues (19, p 83).

The lower chlorine concentrations in the September samples where muriate of potash was applied may have resulted from one or more of three different causes. First may be mentioned the much disputed theory of the outward movement of materials from the plant back to the soil through the roots. For information concerning this theory, the reader is referred to a recent critical review by Thomas (17). Secondly, it is known that chlorine-containing compounds in plants occur not only dissolved in the cell sap, but also as deposits of definite crystals or amorphous compounds in the cell protoplasm (16, p 115). If conditions within the plant during maturation become such as to increase the crystallization and precipitation of chlorine compounds and thus prevent their removal with the sap, the observed decreases in chlorine concentration of the September samples could be accounted for. In the third place, it has been shown by Le Clerc and Breazeale (12) that from 40 to 75 per cent of the chlorine in various crop plants may be washed out by rains and returned to the soil. They point out further that this occurs to a greater extent during maturation than before. Since the rainfall on these plots during September was 6.62 inches, a part of the chlorine losses may have occurred in this way.

Garner and his associates (1) found that a high chlorine content in the leaf of tobacco was accompanied by an increase in its water content. James (11) has recently observed similar increases in the water content of the leaves of potato plants where either muriate of potash or potash manure salts had been applied. If considered casually, these observations would seem to explain the lower chlorine concentrations observed in the studies reported here by assuming that the concentrations were lowered through an increased absorption of water instead of a loss of chlorine. A close examination of Garner's data, however, shows that where muriate of potash was applied the increases in water content are much smaller than the increases in chlorine content. James has reported no chlorine analyses, and hence it is not possible to determine the status of this relation in his studies. On the basis of Garner's data, the hygroscopicity of chlorides can not explain the lower chlorine concentrations in the September samples where muriate of potash was applied, unless it is assumed that (1) the capacity of the plants for absorbing chlorides had been completely satisfied during both August and September, and (2) that the increase in water content did not develop until September. Some indirect evidence has already been presented to support the first assumption, but the latter is unsupported and probably was not operative in the studies reported here.

#### RESIDUAL CHLORINE

It is interesting to note from Figure 1 that although the saps from the untreated ends of plots 3, 5, 6, and 7 contained less chlorine than the saps from the manure and muriate of potash plots, they contained

TABLE 2—Ratio of chlorine to potash in the sap of corn plants grown on plots receiving different fertilizer treatments

Plot No	Treatment <sup>a</sup>	Ratio of chlorine to potash when potash equals 1			
		Aug 29, 1928	Aug 30, 1929	Aug 30, 1930	Aug 27, 1931
2D <sub>1</sub> <sup>b</sup>	O		0 06	0 16	0 15
2D <sub>2</sub>	Ps	0 21	13	15	28
3D <sub>1</sub>	O	49	07	23	20
3D <sub>2</sub>	N, Ps, K	54	33	26	23
4D <sub>1</sub>	Check	41	19	10	11
4D <sub>2</sub>	N(am), Pr	56	21	20	12
5D <sub>1</sub>	O	22	14	25	32
5D <sub>2</sub>	Ps, K	47	33	19	38
6D <sub>1</sub>	O	21	22	27	23
6D <sub>2</sub>	K	48	24	29	38
7D <sub>1</sub>	O	40	15	23	24
7D <sub>2</sub>	N, K	44	26	26	21
8D <sub>1</sub>	O	50	11	21	13
8D <sub>2</sub>	N, Ps	36	22	23	12
9D <sub>1</sub>	O	31	30	21	27
9D <sub>2</sub>	N	33	31	21	43
10D <sub>1</sub>	O	21	22	14	17
10D <sub>2</sub>	M	33	14	10	16
11D <sub>1</sub>	Check	29	34	14	17
11D <sub>2</sub>	M, Pr	40	14	10	16
12D <sub>1</sub>	O	19	18	16	09
12D <sub>2</sub>	M	12	06	09	10
13D <sub>1</sub>	O	28	16	17	07
13D <sub>2</sub>	N, Ps		12	19	13
14D <sub>1</sub>	O		16	14	11
14D <sub>2</sub>	Pt	27	07	04	15

<sup>a</sup> Check, no treatment since 1909; O, no treatment since 1914, N, nitrogen from dried blood, N(am), nitrogen from ammonium sulphate, M, manure, Pr, rock phosphate, Ps, superphosphate, and K, muriate of potash

<sup>b</sup> All plots on range D in 1928, range C in 1929, range B in 1930, and range A in 1931

Concerning the effect of fertilizer treatment on the proportion of chlorine to potash in the sap, the ratios are not entirely consistent. In general, the effect of manure was to lower the chlorine:potash ratio. If sap composition is assumed to be an index of the supply of available nutrients in the soil (14), this indicates that manure supplied the plants with more potash than chlorine. Muriate of potash, on the other hand, usually increased the proportion of chlorine to potash. The increases are relatively small, however, in view of the fact that chlorine and potassium were added in approximately equal amounts.

Attention has already been called to the fact that Garner and his associates (1) found as much chlorine as potash in the stalks and leaves of tobacco plants where muriate of potash was used in the fertilizer. Where no muriate was used, the proportion of chlorine to potash was similar to the ratios given in Table 2. Since the chlorine:potash ratios reported here for corn sap do not even remotely approach the proportions of 1:1 where muriate of potash was used, it would seem that as compared to tobacco the corn plant has a very limited capacity for absorbing and retaining the chloride ion. It must be kept in mind, of course, that Garner's data were obtained from analyses of dehydrated stalk and leaf tissues, and that the ratios reported here represent only the sap fraction that is extractable at 6,500 pounds of pressure per square inch. However, the quantities of potassium and chlorine present in the expressed sap probably give a fair estimate of their relative abundance in the plant,

a heavier withdrawal of the soil's supply of chlorine and therefore a weaker residual effect. Poor fertilization caused inferior crop growth, less absorption of chlorides, and therefore a stronger residual effect.

#### THE CHLORINE POTASH RATIO

Garner and his associates (1) have recently reported that where muriate of potash is used on tobacco, the plant contains approximately as much chlorine as potash. They attach considerable significance to the chlorine:potash ratio because the potassium occurring in the form of chloride is not available for the formation of salts of organic acids, except as replaced by other metals. This is apparently important in tobacco because a high chlorine content as well as a low content of potash salts of organic acids both have an unfavorable effect on the burning quality of the tobacco leaf.

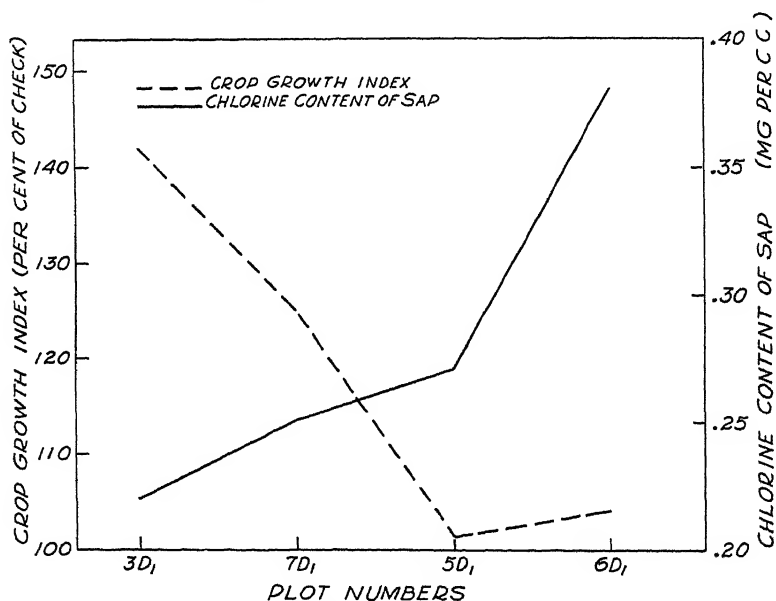


FIGURE 2—Inverse relationship between crop yields and chlorine content of sap of corn plants grown on plots showing residual effects of chlorine applied approximately 15 years ago

Although the physiology of the corn plant is probably very different from that of the tobacco plant in relation to the development of qualities that are of importance in the crop, it may be of interest to compare the amounts of potash and chlorine occurring in the sap of corn plants and to determine the effect of various fertilizers on the chlorine:potash ratio. Accordingly, the ratios have been calculated and are given in Table 2. It is immediately clear from the data that there is much less chlorine than potash in the sap of corn plants. Nearly all of the 1929, 1930, and 1931 ratios lie between 0.1:1.0 and 0.3:1.0, which means that in these three years there was from 10 to 30 per cent as much chlorine in the sap as there was potash. The ratios for 1928 are comparatively high for some reason, many being as high as 0.4 and 0.5.

This differential absorption of chlorides and sulphates is undoubtedly dependent on (1) the structure, chemical composition, and pore spaces of the root-absorbing surfaces, and (2) on the degree of hydration, ionic volume, and degree of dissociation of salts (18). Concerning the latter, it should be noted that the physical and chemical properties of the two ions are distinctly different. Thus, it is well known that the chlorides are not only more soluble than the sulphates, but also that they dissociate to a greater degree. Furthermore, the sulphate ion is approximately three times heavier and larger than the chloride ion. Chloride ions therefore have a greater diffusion velocity than sulphate ions, and, being smaller in volume, have less difficulty in passing through the pore spaces of the root-absorbing membranes. These differences between the two ions are magnified still more by their differential degrees of hydration, for most sulphates attract and hold more molecules of water than chlorides do. This increases the weight and volume and decreases the diffusion velocity of the sulphate ions to a greater extent than for the chloride ions, and imposes a greater handicap on the former than on the latter in passing through the root membranes. It is clear, then, that such influences as solubility, degree of dissociation, ionic volume, and degree of hydration, are all more favorable to the absorption of chloride ions than sulphate ions. Nevertheless, it is unlikely that these differences in the chemical and physical properties of the two ions alone can account for their differential absorption by plants, for Stiles (15) was unable to show any correlation between the rates of diffusion of sulphate and chloride ions into gels and into living cells. It is now generally recognized that the structure and chemical composition of the cell membranes must be taken into consideration as well as the physical and chemical properties of the ions.

The rate of absorption of chlorides and sulphates from soils of pH 5.5 to 7 corresponds to their relative positions in the Hofmeister series for anions, which, in descending order of mobility, is  $\text{OH} > \text{I} > \text{Br} > \text{NO}_3 > \text{Cl} > \text{HPO}_4 > \text{SO}_4$  (2, 18).

#### HYDROGEN-ION CONCENTRATION OF SAP AS AFFECTED BY CHLORINE CONTENT

In a previous report (14, p. 108) on the corn-sap studies now under way at the Virginia station, attention was called to a fairly close relationship between the acidity of the expressed sap and potassium fertilization. In general, the saps from plots where muriate of potash had been applied were most acid (below pH 5.40), those from the manure plots were somewhat less acid, while those from plots where no potash materials had been added were least acid. If these observations are correlated with the chlorine concentrations in the sap (Table 1), it will be found that there is a positive relation between (1) the amount of chlorine supplied by the fertilizer, (2) the chlorine content of the expressed plant sap, and (3) the hydrogen-ion concentration of the sap. The saps from the plots which received muriate of potash were more acid than the others because the large amount of chlorine supplied by the fertilizer caused a large accumulation of chloride ions within the plant. Manure supplied less chlorine, caused a smaller accumulation of chloride ions within the plant, and a lower degree of acidity. Where no chlorine was applied in the fertilizer, there was little accumulation of chloride ions within the

because it is well known that although these elements enter into organic combinations to some extent in the plant, they generally remain dissolved in the cell sap and are therefore subject to extraction with this part of the plant. These differential capacities of corn and tobacco for absorbing chlorides from the soil solution are undoubtedly characteristic of the two species of plants and inherent in them. Harris and his associates (5, 6, 8) found that Egyptian cottons have a larger capacity for absorbing chlorides than do upland cottons. Upon crossing these two types of cotton, they found this character to be heritable, the  $F_1$  progeny were intermediate and the  $F_2$  progeny showed segregation for the ability to absorb selectively either chlorides or sulphates.

#### RELATIVE ABSORPTION OF CHLORIDE AND SULPHATE IONS

A number of investigators have shown that plants absorb the chloride ion much more readily than the sulphate ion. Reference to such work will be limited here to the controlled culture work of Hoagland (9) and Hoagland and Martin (10) at the California station and to the extensive field experiments of Garner and his associates (1) in the United States Department of Agriculture. The results obtained by these men, as well as those of many others, show that the greater absorption of the chloride ion is not limited to a few species of plants, but is quite common throughout the plant kingdom. The only exception that has come to the writer's attention is the larger sulphate content found by Harris and his associates (4, 5, 8) in the leaf-tissue fluids of cotton plants grown on alkali soils in Arizona.

In order to learn whether the general experience of other workers concerning the relative absorption of chloride and sulphate ions was true of the corn plant, an attempt was made to determine the sulphate content of the sap samples gravimetrically. It was soon found, however, that the saps contained such small amounts of sulphates that it would be impossible to make quantitative determinations unless large samples were used. Even the saps from the treated end of plot 4, where 200 pounds of sulphate of ammonia have been applied annually since 1914, were practically devoid of sulphates. Previous analyses for other materials consumed nearly all the sap available, and since neither nephelometric nor micro equipment suitable for determining small amounts of sulphate was available, it was impossible to make quantitative estimations of the sulphate content of the various sap samples. It is clear from this experience and from the fact that 10 c c of sap was sufficient to ascertain the chlorine content of the samples, that the chloride ion is much more abundant in the sap of corn plants than is the sulphate ion. It is well known that some of the sulphur contained in sulphates is transformed into certain proteins and glucosides within the plant, but it is very improbable that such utilization would account for the large differences observed in the chloride and sulphate content of the saps from the various plots. The relative ease with which muriate of potash and farm manure increased the chlorine content of the sap and the failure of sulphate of ammonia to increase the sulphate content appreciably, makes it quite clear that corn plants absorb the chloride ion much more readily than the sulphate.

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plant, and the expressed sap was still less acid. It is clear, then, that because of the relative ease with which the corn plant absorbs and accumulates chloride ions, the amount of chlorides at its disposal is one of the important factors governing the reaction of the cell sap. The soil's supply of sulphates, on the other hand, apparently plays very little part directly in determining the reaction of the sap of corn plants, because of the difficulty with which this ion is absorbed.

The observations reported here with reference to a positive correlation between hydrogen-ion concentration and chlorine content of the sap of corn plants are in accord with those of Harris and his associates (7, 8), who found that the leaf-tissue fluids of Egyptian cottons have a higher chlorine content and are more acid than those of upland cottons.

#### SUMMARY AND CONCLUSIONS

The use of fertilizers containing chlorine increased the chlorine content of the sap of corn plants. The increase is partially proportional to the amount of chlorine supplied by the fertilizer.

Many of the sap samples contained slightly more chlorine in September than in August. Where muriate of potash had been used, there was a slight decrease in chlorine content during September.

Chlorine added in muriate of potash approximately 15 years ago is still exerting a residual effect on the chlorine content of the plant sap. There is a tendency for this residual effect to be inversely proportional to crop yields.

The chlorine supplied by manure 15 years ago is exerting no residual effect at present.

The sap of corn plants contains approximately one-fourth as much chlorine as potash. Muriate of potash increased the chlorine:potash ratio slightly, while manure lowered it slightly.

Fertilizers containing sulphur failed to cause an appreciable accumulation of sulphate ions in the sap of corn plants.

Corn plants absorb the chloride ion much more readily than the sulphate ion.

As the chlorine content of the sap increases, the hydrogen-ion concentration increases also.

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